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Heterominibodies

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The present invention relates to a multifunctional compound, produceable in a mammalian host cell as a secretable and fully functional heterodimer of two polypeptide chains, wherein one of said polypeptide chains comprises, as the only constant region domain of an immunoglobulin heavy chain, the C_H1-domain and the other polypeptide chain comprises the constant C_L-domain of an immunoglobulin light chain, wherein said polypeptide chains further comprise, fused to said constant region domain(s) at least two (poly)peptides having different receptor or ligand functions, wherein further at least two of said different (poly)peptides do not have an intrinsic affinity for one another and wherein said polypeptide chains are linked via said constant region domains. Preferably, said domains, having receptor or ligand function, are in the format of a scFv-fragment. Most preferably, said scFV-fragment comprises the V_H and the V_L regions of the murine anti 17-1A antibody M79, the V_H and the V_L regions of the anti-Lewis Y antibody, as shown in Fig. 6, or the V_H and the V_L regions of the anti-CD3 antibody TR66. Furthermore, the present invention relates to polynucleotides encoding said polypeptide chains as well as vectors comprising said polynucleotides and host cells transformed therewith as well as the use of the above embodiments for the production of said multifunctional compounds. In addition, pharmaceutical and diagnostic compositions are provided, comprising any of the afore-described multifunctional compounds, polynucleotides or vectors. Described is also the use of the afore-mentioned multifunctional compound for preventing and/or treating malignant cell growth, related to malignancies of hemapoietic cells or to solid tumors.

In the mid 1980s, the concept of bispecific antibodies has been developed. By virtue of bispecific antibodies, different antigens, receptors or ligands can be crosslinked, which do not physiologically interact with each other, thus providing novel means of interfering with disease processes, recruiting cytotoxic effector cells to kill target cells, e.g. tumor cells, or virus-infected cells or facilitating the elimination of pathogens from the body.

Small bispecific antibody constructs are commonly thought to have great diagnostic and therapeutic potential. In contrast to bispecific versions of whole immunoglobulin molecules (Merchant, Nature Biotechnology 16 (1998), 677-681) expected to share the in vivo properties and especially the long serum half life of their natural monospecific counterparts, small bispecific antibody constructs due to their reduced molecular weight are preferable for applications requiring improved biodistributional properties. In addition, small bispedific antibodies have been presumed to be producible in significant better yields than bispecific versions based on whole immunoglobulins. Accordingly, several recombinant routes have been developed for the production of such bispecific antibody fragments in order to overcome the low yields of conventional methods (Carter, J. Hematother. 4 (1995) 463-470).

Prior art bispecific antibody fragments usually could not be glycosylated due to their lack of glycosylation sites. Accordingly, production methods have been focused on E.coli as expression host, although functional expression of antibody derivatives in E.coli can be critical, depending on the successful translocation of the corresponding polypeptide chains into the periplasmatic space and on the structural complexity of the recombinant protein. Thus, bispecific single-chain antibodies consisting of four Ig-variable regions on a single polypeptide chain proved to be not expressable as functional proteins in the periplasma of E.coli. In contrast, bispecific single-chain antibodies can be expressed as fully functional recombinant proteins within the secretory pathway of mammalian cells thus allowing the purification from the culture supernatant (Mack, Proc. Natl. Acad. Sci. USA 92 (1995), 7021-7025).

In general, strategies for the expression of bispecific antibodies can be divided into two-host and single-host systems (Carter, J. Hematother. 4 (1995), 463-470). In two-host systems, the two different specificities are separately expressed and purified and subsequently combined in vitro to form bispecific heterodimers. In single-host systems, the bispecific antibody is either expressed in the single chain format or two different polypeptide chains form heterodimers during expression in the same host cell. In principle, single-host systems are more preferable than two-host systems, since the additional in vitro steps required in the two-host systems tend to increase production costs, reduce the yield and limit the attainable purity of the resulting bispecific antibodies. Of course, functional expression of bispecific

antibody derivatives in a suitable single-host system is also preferable to conventional methods relying on non-functional expression followed by complete denaturation of the recombinant protein, and subsequent refolding. Accordingly, efficient methods for the functional expression of bispecific antibodies in single host systems are highly preferable compared to alternative methods.

In addition to the functional expression of bispecific single-chain antibodies in mammalian cells, the only single host system that predominantly produces bispecific antibody fragments and proved to be feasible for production upscaling is the expression of diabodies in the periplasma of E.coli, which is based on the preferential dimerization of two different polypeptide chains (Hollinger, Proc. Natl. Acad. Sci. USA 90 (1993), 6444-6448). As regards the functional expression of small bifunctional antibody constructs comprising at least one non-immunoglobulin part, only mammalian host cells fully meet expression requirements. This is because non-immunoglobulin portions such as the extracellular domains of cellular receptors are often glycosylated and frequently exceed Ig-antigen binding sites in structural complexity. In contrast to mammalian systems, E.coli, yeast or baculovirus systems do not or only partially meet these requirements.

As has been demonstrated by others (Gerstmayer, J. Immunol. 158 (1997), 4584-4590), an appropriate format for the expression of such bifunctional antibody constructs comprising non-immunoglobulin parts in higher host cells is the single chain format. Whereas the single chain format bears a number of significant advantages, it is generally believed that non-immunoglobulin parts comprised therein require the native N-terminus within bifunctional single-chain molecules in order to maintain their function. As a consequence, the Ig-antigen binding site within such a single chain has to be placed at the C-terminus. However, in such constructs the antigen binding activity at the C-terminal position is often lost. This holds true even when the advantageous mammalian expression system is used. Therefore it has to be concluded, that the single-chain approach does not provide a generally applicable format for the functional expression of bifunctional antibody constructs.

Accordingly, the technical problem underlying the present invention was to develop a molecular format for the functional expression of bifunctional antibody constructs that is generally applicable for combinations of any given scFv-antibody fragment optionally in combination with different non-immunoglobulin portions. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a multifunctional compound, produceable in a mammalian host cell as a secretable and fully functional heterodimer of two polypeptide chains, wherein one of said polypeptide chains comprises, as the only constant region domain of an immunoglobulin heavy chain the C_H1-domain and the other polypeptide chain comprises the constant C_L-domain of an immunoglobulin light chain, wherein said polypeptide chains further comprise, fused to said constant region, domains at least two (poly)peptides having different receptor or ligand functions, wherein further at least two cf said different (poly)peptides do not have an intrinsic affinity for one another and wherein said polypeptide chains are linked via said constant domains.

The term "multifunctional compound" as used herein denotes a compound comprising two polypeptide chains, wherein said compound comprises at least two functional domains conferring different functions. Such multifunctional compounds include, e.g., bi-, tri-, or tetraspecific heterominibodies.

The term "domains, having receptor or ligand function" in accordance with the present invention denotes functional domains comprising a three-dimensional structure capable of specifically binding to or interacting with a molecule. Such a molecules can be, but are not limited to, peptides or polypeptides and their post-translational modifications.

The term " different (poly)peptides do not have an intrinsic affinity for one another" means, in accordance with the present invention, that the different (poly)peptides do not naturally tend to associate under physiological conditions such as, for example, V_H and V_L chains do.

Thus, the present invention provides a multifunctional compound comprising two different polypeptide chains wherein efficient heterodimerization of said polypeptide

chains during the expression and the secretion process in mammalian host cells is mediated by the interaction of the above specified constant region domains of immunoglobulin light and heavy chains.

The heterodimerization of constant immunoglobulin domains allows the interaction of two additional different (poly)peptide chains fused thereto without any intrinsic affinity to each other in a single mammalian expression system further allowing relevant post-translational modification and leading to a secretable compound of higher structural complexity.

The domains of the multifunctional compound of the present invention, having receptor or ligand function can be either linked to the C- and the N-terminus of one or both constant immunoglobulin domains. Therefore, the present invention provides multifunctional compounds, which can comprise bi-, tri- or tetrafunctional molecules, wherein each of said receptor or ligand functions can be linked to either the C- or the N-terminus of said constant immunoglobulin domains.
The linkage of said functional domains to the constant immunoglobulin domains can be provided by, e.g. genetic engineering, as described in the examples. Methods for preparing fused and operatively linked polypeptide chains and expressing them in mammalian cells are well-known in the art (e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

As has been detailed above, the solution to the various problems dealt with in the prior art was found to be a molecular format consisting of two different polypeptide chains, wherein one polypeptide chain contains the CH1-domain of an immunoglobulin heavy chain and the other polypeptide chain contains the constant domain of an immunoglobulin light chain, thus mediating efficient heterodimerization of said polypeptide chains during the expression and secretion process in mammalian host cells. This molecular format advantageously provides two N-terminal positions in contrast to the prior art single chain format and proved to be efficiently secreted by mammalian host cells into the culture supernatant, where it is found as fully functional and adequately glycosylated heterodimeric protein that can be easily purified.

Furthermore, the molecular format of the invention additionally provides two C-terminal positions that can be accupied by further protein domains thus resulting in a multifunctional compound carrying more than two different functional entities. In the case that two different (poly)peptides which do not tend to associate under the above recited conditions are fused to the N-termini of the constant region domains, then to each C-terminus of said constant region domains one V_H and V_L region, respectively, may be found. Identifying appropriate heterodimerization domains that meet the above mentioned criteria was no trivial task since previous approaches to obtain preferential heterodimerization in single host expression systems by using heterodimerization domains failed. Heterodimerization domains based on leucine zippers, for example, proved to facilitate the heterodimerization in single host expression systems of polypeptid chains that, although very weakly (Chang, Proc. Natl. Acad. Sci. USA 91 (1994), 11408-11412; Kalandadze, J. Biol. Chem. 271 (1996), 20156-20162) intrinsically tend to heterodimerize with each other like the α and β chains of T-cell receptors or MHC-class II-molecules. However, in cases of two different proteins without any intrinsic affinity to each other, as for example the two different antigen binding sites within heterodimeric bispecific antibodies, jun- and fos-based domains quantitatively produced homodimers instead of heterodimers in single host expression systems (de Kruif, J. Biol. Chem. 271 (1996), 7630-7634). Such jun-and-fos homodimers could be dissociated in vitro, mixed and subjected to conditions facilitating reassociation, which turned out to mainly result in jun-fos-heterodimers (Kostelny, J. Immunol. 148 (1992), 1547-1553). In contrast, the approach of the present invention allows heterodimerization of two different (poly)peptide chains without any intrinsic affinity to each other in a single host expression system wherein said (poly)peptides are fused to said constant region domains. This approach most importantly meets the expression and secretion requirements of mammalian host cells, thus enabling heterodimerization-based bi-and multifunctional compounds, that may be glycosylated and of higher structural complexity, to be functionally expressed and purified from the culture supernatant of the preferably mammalian host cell line.

Bifunctional antibody derivatives described in examples 1-4 (the corresponding embodiments of which are also described further below)and produced according to the molecular format of the invention consist of a scFv-antibody fragment directed against a tumor-associated antigen, e.g. 17-1A or LewisY, and the extracellular part

of cellular receptors (e.g CD80, CD86, CD58, CD54) mainly expressed on antigen presenting cells e.g. dendritic cells and known for their T-cell costimulatory and/or adhesion function. One of these bifunctional antibody derivatives, heterominibody M79scFvCK/CD80CH1 was extensively tested for its functional activity. The recombinant molecule proved to bind to its native target antigen 17-1A on intact cells and was surprisingly found to subsequently provide not only one necessary costimulatory signal to naive T-lymphocytes by virtue of its CD80(B7-1) arm but mediates sufficient costimulation in order to prime naive CD4+-and CD8+-T-cells, that simultaneously receive the first activation signal via an anti-17-1A x anti-CD3 bispecific single chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995), 7021-7025) further desigated M79scFv-antiCD3scFv. The priming events could be clearly demonstrated by switching of the T-cell surface phenotype from that of naive (CD45RA+R0-) to that of primed T-lymphocytes (CD45RA-RO+) and could be confirmed by determination of characteristic cytokines in the T-cell supernatant. Exclusive secretion of γ but not IL-5 by in vitro primed CD4+-T-lymphocytes furthermore interestingly demonstrated, that the heterominibody M79scFvCK/CD80CH1 selectively mediates the priming and differentiation of CD4+T-cells that express the TH1-phenotype, which is envisaged to advantageously augment the cellular immune response against tumor cells in vivo. Other bifunctional tumorspecific B7(CD80 or C 86) constructs described in the literature, have so far never been shown to provide sufficient costimulation for the priming of naive T-cells (Gerstmayer, J. Immunol. 158 (1997), 4584-4590; Challita-Eid, J. Immunol. 160 (1998), 3419-3426). It is envisaged that such costimulatory heterominibody constructs can be applied in vivo alone or in combination with a bispecific antibody which provides the primary T-cell activation signal independent of the clonotypic T-cell receptor. It is further envisaged that such a combination can be attained by structurally combining features of both molecules within one multifunctional compound according to the molecular format of the invention as described in example 7 and shown in figure 23.

In a preferred embodiment, the multifunctional compound of the present invention comprises at least three functional domains, having receptor or ligand function, providing a tri-specific heterominibody.

The present invention also relates, in a further preferred embodiment, to the multifunctional compound of the present invention, wherein at least two domains, having receptor or ligand function, are N-terminally linked to said constant C_H1 or C_L domains.

In yet another preferred embodiment, the invention relates to the multifunctional compound of the invention, wherein at least one of said domains, having receptor or ligand function, is in the format of a scFV-fragment or a functional part thereof.

The multifunctional compound of the invention, wherein at least one of said domains, having receptor or ligand function, is a T-cell co-stimulatory ligand, an antigen binding region specific for a tumor associated antigen, or a proteinaceous compound providing the primary activation signal for T-cells, is still another preferred embodiment of the invention.

Adequate activation resulting in priming of naive T-cells is critical to primary immunoresponses and depends on two signals derived from professional APCs (antigen-presenting cells) like dendritic cells. The first signal is antigen-specific and normally mediated by stimulation of the clonotypic T-cell antigen receptor that is induced by processed antigen presented in the context of MHC class-I or MHC class-II molecules. However, this primary stimulus is insufficient to induce priming responses of naive T-cells, and the second signal is required which is provided by an interaction of specific T-cell surface molecules binding to co-stimulatory ligand molecules on antigen presenting cells, further supporting the proliferation of primed T-cells. The term "T-cell co-stimulatory ligand" therefore denotes in the light of the present invention molecules, which are able to support priming of naive T-cells in combination with the primary stimulus and include, but are not limited to, members of the B7 family of proteins, including B7-1 C CD80) and B7-2 (CD86).

An antigen binding region specific for a tumor associated antigen denote antibody fragments directed against tumor associated antigen known in the art, e.g. 17-1A or Lewis Y, Muc-1, erbB2 or s-Tn.

In the light of the present invention, "proteinaceous compounds" providing the primary activation signal for T-cells" can comprise, but are not limited to, anti-CD3-

svFv fragments, anti-T-cell receptor svFv fragments or superantigens. Superantigens directly bind to certain subfamilies of T-cell receptor variable regions in an MHC-independent manner thus mediating the primary T-cell activation signal.

Moreover, in yet another preferred embodiment, the invention relates to the multifunctional compound of the invention, wherein said scFv fragment or said functional part thereof comprises the V_H and the V_L regions of the murine anti 17-1A antibody M79 (Göttlinger, Int. J. Cancer 38 (1986), 47), the V_H and the V_L regions of the anti-Lewis Y antibody as shown in Figure 6 or the V_H and/or the V_L regions of the anti-CD3 antibody TR66 (Traunecker, EMBO J. 10 (1991) 3655).

In a more preferred embodiment, the invention relates to the multifunctional compound of the invention, wherein the T-cell co-stimulatory ligand is a cell surface molecule or a fragment thereof expressed on antigen-presenting cells (APC).

In an even more preferred embodiment, the multifunctional compound of the invention comprises an antigen-presenting cell, which is a dendritic cell.

Furthermore, in a most preferred embodiment, the present invention relates to the multifunctional compound of the invention, wherein the cell surface molecule on an APC is a T-cell co-stimulatory factor like B7-1 (CD80) or B7-2 (CD86), or adhesion proteins like LFA-3 (CD58), ICAM-1 (CD54), ICAM-2 or ICAM-3 or like the CD137-ligand.

The multifunctional compound of the invention, wherein said constant domain of an immunoglobulin light chain is of the κ type, is another subject matter of the present invention. Optionally, the constant domain of an immunoglobulin light chain can be of the λ type.

In yet another embodiment, the present invention relates to the multifunctional compound of the invention, wherein said constant immunoglobulin domains and the above-described functional receptor-ligand domains are connected by a polypeptide linker.

This polypeptide linker, disposed between the immunoglobulin domains and the functional receptor-ligand domains preferably comprises plural, hydrophilic, peptide-bonded amino acids that are covalently linked to these domains.

In a more preferred embodiment, said polypeptide linker comprises an Ig-hinge region or a plurality of glycine, alanine and/or serine.

In a particularly preferred embodiment, said Ig-hinge region is an IgG hinge region.

In a most preferred embodiment, the IgG hinge region is the upper hinge region of human IgG_3.

In another preferred embodiment, the present invention relates to the multifunctional compound of the invention, wherein said C_H1 domain further comprises a histidine tag, GST, a Staphylococcus protein A tag, Lex A, a FLAG tag or a MYC-tag.
These additional sequences, capable of selective binding to a solid support or to be used for purification purposes, can be either full-length polypeptide sequences or fragments thereof. Due to the fact that these tags are fused to the CH1 domain, the complete multifunctional compound can conventionally be isolated.

Apart from that, the present invention also relates to a polynucleotide encoding one and/or two polypeptide chains of the multifunctional compound as defined herein above.
Said polynucleotide may be fused to suitable expression control sequences known in the art to ensure proper transcription and translation of the polypeptide chains. Said polynucleotide may be, e.g., DNA, cDNA, RNA or synthetically produced DNA or RNA or a recombinantly produced chimeric nucleic acid molecule comprising any of those polynucleotides either alone or in combination. Preferably said polynucleotide is part of a vector. Such vectors may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Preferably, the polynucleotide of the invention is operatively linked to expression control sequences allowing expression in eukaryotic cells. Expression of said polynucleotide comprises transcription of the

polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory seuqences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in mammalian host cells comprise the CMV-, SV40, RSV-promoter (Rous sarcome virus), human elongation factor 1α-promoter, CMV-enhancer or SV40-enhancer. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL), pEF-DHFR (Mack, PNAS 92 (1995), 7021-7025). Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting mammalian host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the polypeptide of the invention may follow.

A vector comprising at least one of the above-mentioned polynucleotide is another subject matter of the present invention.
The vector of the present invention may be, e.g., a plasmid, cosmid, virus, bacteriophage or another vector used conventionally in genetic engineering, and may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions.

In another embodiment, the present invention relates to a mammalian host cell comprising at least one vector of the present invention.

In a preferred embodiment, the mammalian host cell of the invention is a CHO cell or a myeloma cell.

Furthermore, the present invention relates to a method of producing the multifunctional compound of the invention, this method comprising culturing the host cell of the present invention under conditions that allow the synthesis of said multifunctional compound, and recovering said multifunctional compound from the culture.

Thus, the present invention allows the recombinant production of multifunctional compounds comprising sites and domains of T-cell co-stimulating ligands, antigen binding regions specific for a tumor associated antigen or proteinaceous compounds providing first activation signals for T-cells. As is evident from the foregoing, the invention provides a large family of multifunctional comprising receptor-ligand functions for any use in therapeutic and diagnostic approaches.
It will be apparent to those skilled in the art that the multifunctional compounds of the invention can be further coupled to other moieties for, e.g. drug targeting or diagnostic imaging applications. Such coupling may be conducted chemically after expression of the multifunctional compound or of the expression of the polypeptide chains of the invention or the coupling product may be engineered into the polynucleotides of the invention as discussed herein above. The polynucleotides are then expressed in a suitable host system and the expressed multifunctional compounds are collected and purified, if necessary.

In another embodiment, the present invention relates to a pharmaceutical composition comprising the multifunctional compound of the present invention, the polynucleotide of the invention, and/or the vector of the invention and, optionaly, a proteinaceous compound capable of providing the primary activation signal for T-cells and a pharmaceutically acceptable carrier and/or the diluent and/or excipient.
According to the present invention, proteinaceous compounds capable of providing the primary activation signal for T-cells in pharmaceutical and/or diagnostic compositions are monospecific or bispecific antibodies interacting with the CD-3-complex, the T-cell receptor as well as compounds including a superantigen.

A diagnostic composition comprising the multifunctional compound of the invention, the polynucleotide of the invention, and/or the vector of the invention and, optionally, a proteinous compound capable of providing the primary activation

signal for T-cells and, optionally, suitable means for detection, is also a subject matter of the present invention.

In another preferred embodiment, the present invention relates to the use of the multifunctional compound of the invention, the polynucleotide of the invention and/or the vector of the invention for the preparation of a pharmaceutical composition for preventing and/or treating a malignant cell growth.

In a particularly preferred embodiment, the present invention relates to the above-described use, wherein malignant cell growth is related to malignancies of hemapoietic cells or to solid tumors.

In an even more preferred embodiment, the present invention relates to the use of the invention, wherein said malignancies of hematopoietic cells are lymphomas or leukemias.

In a most preferred embodiment, however, the present invention relates to the use of the present invention, wherein said solid tumors are carcinomas, melanomas or sarcomas.

A kit comprising the multifunctional compound of the invention and, optionally, a proteinaceous compound capable of providing the primary activation signal for T-cells, is also the subject matter of the present invention.

In another preferred embodiment, the present invention relates to the pharmaceutical composition of the invention, to the diagnostic composition of the invention or to the kit of the invention, wherein the proteinaceous compound capable of providing the primary activating signal for T-cells is a bispecific antibody interacting with the T-cell antigen CD3.

The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier, diluents and excipients. Examples of suitable pharmaceutical carriers, diluents and excipients are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water

emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Proteinaceous pharmaceutically active matter may be present in amounts between 1 ng and 10 mg per dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patent depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently.

The Figures show:

Figure 1: Molecular design of Heterominibody M79scFvCH1/CD80CK shown on the protein level. CH1 and CK indicates the first constant domain of human IgG1 heavy chain and the constant region of the human Ig-kappa light chain/ respectively, that together form the heterodimerization unit covalently joined together by a disulfide bridge (S-S). VH indicates the Ig-heavy chain variable region and VL the Ig-light chain variable region.

(B-J) Molecular design of Heterominibodies (B) M79scFvCK/CD80CH1 (C) M79scFvCH1/CD54CK (D) M79scFvCK/CD54CH1 (E) M79scFvCH1/CD58CK (F) M79scFvCK/CD58CH1 (G) M79scFvCH1/CD86CK (H) M79scFvCK/CD86CH1 (I) antiLeyscFvCH1/CD80CK (J) antiLeyscFvCK/CD80CH1 shown on the protein-level. For details see legend of Fig 1(A)

Figure 2: DNA-sequence designated CTI that was cloned into the multiple cloning site of the Bluescript KS vector (GenBank accession number X52327) by using the restriction sites XbaI and SalI in order to increase the number of possible cloning sites. CTI-derived restriction enzyme cleavage sites are shown.

Figure 3: Fig 3 Molecular design of DNA-fragments encoding the single polypeptide chains of Heterominibodies (A) M79scFvCH1/CD80CK (B) M79scFvCK/CD80CH1 (C) M79scFvCH1/CD54CK (D) M79scFvCK/CD54CH1 (E) M79scFvCH1/CD58CK (F) M79scFvCK/CD58CH1 (G) M79scFvCH1/CD86CK (H) M79scFvCK/CD86CH1 (I) antiLeyscFvCH1/CD80CK (J) antiLeyscFvCK/CD80CH1

Note that some Heterominibodies share the same polypeptide chain; in these cases the corresponding polypeptide chains are only shown once. Symbols are used as in figures 1(A) and 1(B), the expression

vector pEF-DHFR or pEF-ADA used for cloning and expression of individual chains is indicated. LewisY is abbreviated as Ley.

Figure 4: ELISA-analysis of cell-culture supernatants obtained from CHO cells transfected with the expression plasmid pEF-DHFR-M79 scFv-CK pEFADA/CD80-CH1 after different amplification steps and after subcloning. 96 well ELISA plates were incubated with 50µl of soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant was added as indicated. Detection was performed by a human CK biotin labeled antibody (Pierce, Cat No. 31780) diluted 1:1000 and peroxidase conjugated Avidin (Dako, Hamburg Cat No. P0347) diluted 1:1000. As negative control, well were incubated with phosphate buffered saline. The ELISA was developed by ABTS-substrate solution as described in example 2.1. OD-values were measured at 405nm using an ELISA reader.

Figure 5: FACS analysis of Heterominibody M79 scFv-CK/CD80-CH1 binding to 17-1A transfected CHO cells. 200.000 17-1A transfected CHO cells (see example 5.1) were incubated for 30 minutes with several dilutions of Heterominibody M79scFv-CK/CD80-CH1 ranging from 4µg/ml to 3,9ng/ml. Thereafter, cells were washed twice in PBS and incubated for 30 minutes with a R-Pycoerythrin conjugated murine anti-human CD80 antibody (Becton Dickinson Immunocytometry Systems, San Jose CA, USA, Cat No. 340294). After two final washing steps in PBS, cells were then analyzed by flowcytrometry (FACS-SCAN Becton Dickinson Immunocytometry Systems, San Jose CA, USA).

Figure 6: Fig 6 DNA- as well as amino acid- sequence of antiLewisY scFv-fragment carrying a leader sequence at its N-terminus. The nucleotides from 68 to 394 encode for the Ig-light chain variable region, the nucleotides from 440 to 793 encode for the Ig-heavy chain variable domain. The restriction enzyme cleavage sites important for cloning are indicated.

Figure 7: ELISA-analysis of cell-culture supernatants obtained from two different CHO-cell-transfectants, double-transfected with either the expression plasmids pEF-DHFR-anti-Lewis Y-scFv-CK and pEFADA-CD80-CH1 or with pEFDHFR-CD80-Ck and pEFADA anti-Lewis Y-scFv-CH1 after the first gene-amplification step, respectively. 96 well ELISA plates were incubated with 50µl of soluble Lewis Y-BSA conjugate (30µg/ml) per well. Subsequently pure cell-culture supernatant thereof was added as indicated. Detection was performed by a CD80-specific monoclonal antibody (Immunotech, Cat No. 1449) diluted 1:1000 and a peroxidase conjugated goat anti-Mouse IgG (Fc)-antibody (Dianova Hamburg) diluted 1:5000. As negative control, wells were incubated with phosphat buffered saline. The ELISA was developed by ABTS -substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader. LeY is used as abbreviation of anti-Lewis Y scFv.

Figure 8: ELISA-analysis of cell-culture supernatants obtained from two different CHO-cell-transfectants, double-transfected with either the expression plasmids pEF-DHFR-anti-Lewis Y-scFv-CK and pEFADA-CD80-CH1 or with pEFDHFR-CD80-Ck and pEFADA anti-Lewis Y-scFv-CH1 after the first gene-amplification step, respectively. 96 well ELISA plates were incubated with 50µl of BSA-free anti-his tag antibody (25µg/ml) per well. Subsequently pure cell-culture supernatant was added as indicated. Detection was performed by a biotin-labeled anti-human CK antibody (Pierce, Cat No .31780) diluted 1:1000 and peroxidase conjugated Avidin (Dako, Hamburg, Cat No. P0347) diluted 1:1000. As negative control, wells were incubated with phosphat buffered saline. The ELISA was developed by ABTS -substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader. Ley is used as abbreviation of anti-Lewis Y scFv.

Figure 9: ELISA on cell-culture supernatant of both versions of Heterominibody CD80, CD86, CD58, CD54 (specific detection)

Binding to the 17-1A-antigen was analyzed using recombinant 17-1A-antigen obtained by stable expression in CHO-cells as described (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025 and example 4.4) The antigen was immobilized on 96 well U bottom ELISA plates (nunc maxisorb) at a concentration of 50µg/ml phosphate buffered saline PBS. Coating was carried out at 4°C for 12 hours with 50µl followed by washing once with (PBS) 0,05%Tween. The ELISA was then blocked for 1 hour with PBS/3%bovine serum albumin (BSA) and washed again once. Subsequently, the cell-culture supernatant was added undiluted and at several dilutions (pure, 1:2, 1:4, 1:8, 1:16, 1:32) and incubated for 2 hours. Specific detection was dependent on the type of costimulatory proteins associated with the different Heterobody version. Specific antibodies anti CD54, anti CD58, anti CD80 and anti CD86 were used all diluted 1:1000 (for details see table 4.4.) . After three times of washing with PBS 0,05% Tween20, a polyclonal peroxidase-conjugated goat anti-mouse IgG-antibody (Fc-specific) (Dianova Hamburg) diluted 1:5000 was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the ABTS substrate as described in Example 4.4. As negative control the plates were incubated with PBS instead of Heterominibody constructs. The colored precipitate was measured at 405 nm using an ELISA-reader.

Figure 10: ELISA on cell-culture supernatant of both versions of Heterobody CD80, CD86, CD58, CD54 (anti human Ckappa detection)

An anti-His-tag-antibody (DIANOVA; Hamburg Cat No. DIA 910) diluted 1:40 was coated to 96-well plates as described above. Supernatants of all Heterominibody versions were added pure and in dilutions 1:2, 1:4, 1:8. A biotinylated anti-human Ckappa antibody (Pierce, Cat. No. 31780) followed by peroxidase-conjugated streptavidin (1:1000) (Dako, Hamburg, Cat No. P0347) was used for detection of bound Heterominibodies (see Table 4.4). After four times of washing with PBS 0,05% Tween20, the ELISA was finally

developed by adding the ABTS substrate as described in Example 4.4. As negative control the plates were incubated with PBS instead of Heterominibody constructs. The colored precipitate was measured at 405 nm using an ELISA-reader.

Fig. 11 FACS-Control of the CHO cells after transfection with 17-1A.
The expression of transmembrane 17-1A was increased by stepwise gene amplification induced by subsequent addition of increasing concentrations of the DHFR inhibitor MTX to a final concentration of 500nM, with the concentration steps in between 20nM and 100nM. These cells were tested for membrane expression of 17-1A by flow cytometry at a concentration of 10µg/ml of the 17-1A-specific antibody M79 (Göttinger, In6. J. Cancer 38(1986) 47-53) followed by a FITC-labeled polyclonal Goat Anti Mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. As negative control untransfected CHO cells were used whereas the 17-1A-positive human gastric cancer celline Kato, obtained from ATCC served as positive control.

Figure 12: BrdU-incorporation of CD4+CD45RA+T-cells after stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-antiCD3scFv. After 3 days of stimulation the cells were incubated with BrdU for 14 hours. The assay was performed as recomended in the product description by Boehringer Mannheim Cat.No. 1647229. The OD values were measured at 450nm using an ELISA reader.

Abbreviations:
without CHO max = without 17-1A-transfected CHO-cells plus 250 ng/ml bispecific single-chain antibody M79scFv-anti-CD2scFv plus 500 ng/ml M79scFv CK/CD80CH1-heterominibody

without CHO Bimax = without 17-1A-transfected CHO-cells plus 250 ng/ml bispecific single-chain antibody M79scFv-anti-CD2scFv

PBLsMBmaxBimax = 17-1A-transfected CHO-cells plus unseperated mononuclear cells from peripheral blood plus 250 ng/ml bispecific single-chain antibody M79scFv-anti-CD3scFv plus 500 ng/ml M79scFv CK/CD80 CH1-heterominibody

PBLBimax = 17-1A-transfected CHO-cells plus unseperated mononuclear cells from peripheral blood plus 250 ng/ml bispecifc single-chain antibody M79scFv-anti-CD2scFv

PBL neg = negative control consisting of 17-1A-transfected CHO-cells plus unseparated mononuclear cells from peripheral blood

Figure 13: Percentage of CD4+ CD45RA+CD45R0- T-cells after 3 days of stimulation of CD4+CD45RA+T-cells with Heterominibody M79CK/CD80CH1 and/or M79scFv-antiCD3 analyzed by FACS
CD45RA and CD45RO expression levels were analyzed by flowcytometry after 3 days of stimulation of CD4+CD45RA+T-cells with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250, 50, 10, 2 ng/ml M79scFv-antiCD3 scFv. Figure 5.4.1 shows the percentage of CD4+CD45RA-CD45RO+ T-cells of all gated cells. 100.000 cells were washed once with PBS and incubated for 30 minutes with a R-Phyco-erythrin conjugated anti-human CD45RA antibody (2H4 Coulter) diluted 1:50 and with a FITC conjugated anti-human CD45RO antibody (UHCL-1 DAKO Hamburg) diluted 1:50 and washed again once with PBS. As positive control PBMCs were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3scFv. For negative controls unstimulated PBMC, and purified CD4+CD45RO- T-cells stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv without 17-1A transfected CHO cells. Abbreviations see legend Fig. 12.

Figure 14: Percentage of CD4+ CD45RA-CD45R0+ T-cells after 6 days of stimulation of CD4+CD45RA+T-cells with Heterominibody M79CK/CD80CH1 and/or M79scFv-antiCD3 scFv analyzed by FACS
CD45RA and CD45RO expression levels were analyzed by flowcytometry after 6 days of stimulation of CD4+CD45RA+T-cells with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250, 50, 10, 2

ng/ml M79scFv-antiCD3 scFv. Figure 5.4.1 shows the percentage of CD4+CD45RA-CD45RO+ T-cells of all gated cells. 100.000 cells were washed once with PBS and incubated for 30 minutes with a R-Phycoerythrin conjugated anti human CD45RA antibody (2H4 Coulter) diluted 1:50 and with a FITC conjugated anti human CD45RO antibody (UHCL-1 DAKO Hamburg) diluted 1:50 and washed again once with PBS. As positive control PBMCs were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv. For negative controls unstimulated PBMC, and purified CD4+CD45RO- T-cells were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv without 17-1A transfected CHO cells. Abbreviations see legend Fig. 12.

Figure 15: γ-IFN ELISA analysis of CD4+ CD45RA+T-cells after 3 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. Cell-culture supernatant was diluted 1:5 prior to ELISA-analysis, the γ standard (supplied with the test-kit) was used as positive control. As negative control, wells were incubated with cell-culture-medium. The ELISA was performed according to manufacturers' manual recomondation (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat.No. 80-3932-00) and developed by ABTS -substrate solution as described in example 2.1 The OD-values were measured at 405nm using an ELISA reader. Abbreviations see legend Fig. 12.

Figure 16: γ-IFN ELISA analysis of CD4+ CD45RA+T-cells after 6 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. Cell-culture supernatant was diluted 1:5 prior to ELISA-analysis, the γ-IFN standard (supplied with the test-kit) was used as positive control. As negative control, wells were incubated with cell-culture-medium. The ELISA was performed according to manufacturers' manual (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat.No. 80-3932-00) and developed by ABTS -

substrate solution as described in example 2.1 The OD-values were measured at 405nm using an ELISA reader. Abbreviations see legend Fig. 12.

Figure 17: IL-5 ELISA analysis of CD4+ CD45RA+T-cells after 3 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. Cell-culture supernatant was analyzed undiluted, the IL-5 Standart (supplied with the test-kit) was used as positive control. As negative control, wells were incubated with cell-culture-medium. The ELISA was performed according to manufacturers' manual (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat.No. 80-5025-00) and developed by ABTS -substrate solution as described in example 2.1 The OD-values were measured at 405nm using an ELISA reader. Abbreviations see legend Fig. 12

Figure 18: IL-5 ELISA analysis of CD4+ CD45RA+T-cells after 6 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. Cell-culture supernatant was analyzed undiluted, the IL-5 standard (supplied with the test-kit) was used as positive control. As negative control, wells were incubated with cell-culture-medium. The ELISA was performed according to manufacturers' manual (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat.No. 80-5025-00) and developed by ABTS -substrate solution as described in example 2.1 The OD-values were measured at 405nm using an ELISA reader. Abbreviations see legend Fig. 12.

Figure 19: BrdU-incorporation of CD8+CD45RA+cells after 3 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. After 3 days of stimulation cells were incubated with BrdU for 14 hours. The assay was performed as recomended in the product description by Boehringer Mannheim Cat.No. 1647229. The OD values were measured at 450nm using an ELISA reader. Abbreviations see legend Fig. 12.

Figure 20: Percentage of CD8+ CD45RA-CD45R0+ T-cells after 4 days of stimulation of CD8+CD45RA+T-cells with Heterominibody M79CK/CD80CH1 and/or M79scFv-antiCD3 scFv analyzed by FACS CD45RA and CD45RO expression levels were analyzed by flowcytometry after 4 days of stimulation of CD8+CD45RA with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250, 50, 10, 2 ng/ml M79scFv-antiCD3 scFv. Figure 5.4.1 shows the percentage of CD8+CD45RA-CD45RO+ T-cells of all gated cells. 100.000 cells were washed once with PBS and incubated for 30 minutes with a R-Phycoerythrin conjugated anti-human CD45RA antibody (2H4 Coulter) diluted 1:50 and with a FITC conjugated anti-human CD45RO antibody (UHCL-1 DAKO Hamburg) diluted 1:50 and washed again once with PBS. As positive control PBMCs were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv. For negative controls unstimulated PBMC, and purified CD8+CD45RO- T-cells were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv without 17-1A transfected CHO cells. Abbreviations see legend Fig. 12.

Figure 21: Percentage of CD8+ CD45RA-CD45R0+ T-cells after 6 days of stimulation of CD8+CD45RA+T-cells with Heterominibody M79CK/CD80CH1 and/or M79scFv-antiCD3scFv analyzed by FACS CD45RA and CD45RO expression levels were analyzed by flowcytometry after 6 days of stimulation of CD8+CD45RA+T-cells with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250, 50, 10, 2 ng/ml M79scFv-antiCD3 scFv. Figure 5.4.1 shows the percentage of CD8+CD45RA-CD45RO+ T-cells of all gated cells. 100.000 cells were washed once with PBS and incubated for 30 minutes with a R-Phyco-erythrin conjugated anti human CD45RA antibody (2H4 Coulter) diluted 1:50 and with a FITC conjugated anti-human CD45RO antibody (UHCL-1 DAKO Hamburg) diluted 1:50 and washed again once with PBS. As positive control PBMCs were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml

M79scFv-antiCD3 scFv. For negative controls unstimulated PBMC, and purified CD8+CD45RO- T-cells were stimulated with 500ng/ml Heterominibody M79CK/CD80CH1 and/or 250 ng/ml M79scFv-antiCD3 scFv without 17-1A transfected CHO cells. Abbreviations see legend Fig. 12.

Figure 22: TNF-α ELISA analysis of CD8+ CD45RA+T-cells after 4 days of stimulation with Heterominibody M79CK/CD80CH1 and/or M79scFv-anti CD3 scFv. Cell-culture supernatant was analyzed undiluted, the TNF-α standard (supplied with the test-kit) was used as positive control. As negative control, wells were incubated with cell-culture-medium. The ELISA was performed according to manufacturers' manual (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat.No. 80-3933-00) and developed by ABTS -substrate solution as described in example 2.1 The OD-values were measured at 405nm using an ELISA reader. Abbreviations see legend Fig. 12.

Fig 23 Molecular design of Heterominibody M79scFv-CK-antiCD3scFv/CD80CH1 shown on the protein level. CH1 and CK indicates the first constant domain of human IgG1 heavy chain and the constant region of the human Ig-kappa light chain/ respectively, that together form the heterodimerization unit covalently joined together by a disulfide bridge (S-S). VH indicates the Ig-heavy chain variable region and VL the Ig-light chain variable region.

Fig.24: Design of various bifunctional CD80-scFv-constructs showing the construction elements on the protein-level. VH indicates the variable region of the Ig-heavy chain, VL that of the Ig-light chain.

Fig.25: Design of various bifunctional CD80-scFv-constructs showing the construction elements on the DNA-level as well as the restriction enzym cleavage sites used.

Fig 26.: ELISA-analysis of the cell-culture supernatant obtained from CHO cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(VL/VH)

including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with 50µl of soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant dilutions thereof were added as indicated. Detection was performed by a murine IgG1 anti His-tag antibody (dianova, Hamburg) diluted 1:1000 and a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova,Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl .Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control. As negative control, wells were incubated with phosphat buffered saline. The ELISA was developed by ABTS -substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader.

Fig. 27: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(VL/VH) including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Detection was performed by a murine IgG1-anti CD80 antibody diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova,Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025). was used as positive control and detected as described in Fig 26. As negative control, wells were incubated with phosphat buffered saline. The ELISA was developed by an ABTS -substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader.

Fig. 28: ELISA-analysis of the purified recombinant CD80-M79scFv(VL/VH)-construct with a short $(Gly_4Ser_1)_1$ linker obtained by purification from cell-culture supernatant using a Ni-NTA-column as described (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025). 96 well ELISA plates were coated overnight at 4°C with pure eluate from the Ni-NTA-column and dilutions thereof as indicated. Subsequently bound recombinant protein was detected by a murin IgG1-anti CD80 antibody diluted 1:1000 or by a murine IgG1-anti His-tag antibody (dianova, Hamburg) diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova,Hamburg) respectively diluted 1:5000. As

negative control wells were coated overnight at 4°C with 3% BSA in phosphate buffered saline. The ELISA was developed by an ABTS -substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader.

Fig. 29: ELISA-analysis of the cell-culture supernatant obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv(VH/VL) including the coding sequence of the short $(Gly_4Ser_1)_1$ linker. 96 well ELISA plates were incubated with soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Detection was performed by a murine IgG1-anti CD80 antibody diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti-mouse IgG (Fc) antibody (dianova,Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control and detected as described in Fig 26. As negative control wells were incubated with phosphat buffered saline. The ELISA was processed by an ABTS-substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader.

Fig. 30: DNA-sequence of the double-stranded oligonucleotide designated ACCGS15BAM with single-stranded overhangs compatible with those of restriction enzymes BspEI and BamHI. Amino acids encoded by the nucleotide sequence are shown.

Fig. 31: ELISA-analysis of the cell-culture supernatant and of its dilutions obtained from CHO-cells transfected with the expression plasmid pEF-DHFR+CTI+CD80-M79scFv (VH/VL) including the coding sequence of the long $(Gly_4Ser_1)_3$ linker. 96 well ELISA plates were incubated with 50µl soluble 17-1A antigen (50µg/ml) per well. Subsequently pure cell-culture supernatant and dilutions thereof were added as indicated. Bound protein was detected by a murine anti His-tag antibody (dianova,Hamburg) diluted 1:1000 followed by a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) antibody (dianova,Hamburg) diluted 1:5000. The anti-17-1A/anti-CD3 bispecific-single-chain antibody antibody (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was used as positive control. As negative control wells

were incubated with phosphat buffered saline. The ELISA was developed by an ABTS-substrate solution as described in example 2.1. The OD-values were measured at 405nm by an ELISA reader.

Fig 32 Molecular design of DNA-fragments encoding the single polypeptide chains of Heterominibody M79scFv-CK-anti-CD3scFv/CD80CH1. Symbols are used as in figures 1(A) and 1(B), the expression vector pEF-DHFR or pEF-ADA used for cloning and expression of individual chains is indicated, Gly_4Ser_1 indicates a S-amino acid Glycin-Serin-Linker

The examples illustrate the invention.

Example 1

Example 1.1 M79scFvCH1/CD80CK Heterominibody

A protein was constructed that connects the single-chain Fv fragment (scFv) of the murine anti 17-1A antibody M79 (Göttlinger, Int. J. Cancer 38 (1986) 47-53) with the extracellular domains of human CD80 by virtue of the heterodimeric association of the immunoglobulin domains CH1 from the human γ1 heavy chain and Ck, the constant region of the human kappa light chain. For this purpose the M79scFv was connected to the human CH1 and the extracellular part of human CD80 was joined to human Ckappa, the resulting polypeptide encoding chains were inserted into separate expression vectors and both transfected into the same mammalian host cell line resulting in the CD80 Heterominibody displayed in figure1 In the following example the construction procedure is being described step by step.

Example 1.1.1 Construction of the CD80-CK chain

First the CD80-Ckappa chain was assembled. The Ck DNA fragment was obtained by PCR using specific 5´and 3´primers. The cDNA template for this PCR was prepared by reverse transcription of the total RNA prepared from human peripheral blood mononuclear cells according to standard procedures. (Sambrook,Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, cold Spring Habour, New York (1989)). The 5`HuCKBspE1 primer introduces the restriction cleavage sites BspE1 and BsiW1 as well as the hinge region of IgG3 (5`HuCKBspE1: 5`AAT TCC GGA ACC CCG CTG GGT GAC ACC ACC CAC ACC CGT ACG GTG GCT GCA CCA TCT GTC TTC 3`) ,the 3`HuCKSalNOT primer introduces the cleavage sites Sal1 and Not1 (3`HuCKSalNOT: 5`ATA AGA ATG CGG CCG CGT CGA CTA ACA CTC TCC CCT GTT GAA GCT C-3`). The CD80 fragment was obtained by polymerase chain reaction (PCR) using specific oligonucleotide primers complementary to the 5´ and 3´ ends of the nucleotide sequence encoding the extracellular part of CD80 (Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.). These primers also introduce an EcoRI and a BspEI cleavage site (5´CD80 Primer: 5´GCA GAA TTC ACC ATG GGC CAC ACA CGG AGG CAG 3´; 3´CD80 Primer: 5´TGG TCC GGA GTT ATC AGG AAA ATG

CTC TTG CTT G 3′) The cDNA template used for this PCR was prepared by reverse transcription of the total RNA prepared from the Burkitt-lymphoma cell line Raji according to standard procedures (Sambrook,Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, New York (1989)).

The CD80 costimulatory protein belongs to the Ig superfamily. It is a heavily glycosylated protein of 262 amino acids. A more detailed description was published by Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.

The CD80-Ckappa chain was cloned in several steps using the already existing vector pEF-DHFR-CTI-CD80-M79scFv. This vector was made as follows.
First a poly-linker designated CTI was inserted into the Bluescript KS vector (GenBank® accession number X52327) using the restriction enzyme cleavage sites XbaI and SalI (Boehringer Mannheim). The introduction of the polylinker CTI provided additional cleavage sites as well as the sequence encoding a $(Gly_4Ser_1)_1$ linker, a six-amino acid histidine tag and a stop codon as shown in figure 2 The vector BluescriptKS-CTI was prepared by cleavage with the restriction enzymes EcoRV and XmaI (Boehringer Mannheim and New England Biolabs) in order to ligate it (T4 DNA, Ligase Boehringer Mannheim) with the M79scFv fragment cleaved by EcoRV and BspEI (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025) The resulting vector BluescriptKS-CTI-M79scFv again was cleaved with EcoRI (Boehringer Mannheim) and BspEI in order to insert the CD80 PCR-DNA-fragment obtained as described above and cleaved with the same enzymes. Subsequently, the whole CD80-M79scFv (VL/VH) DNA fragment was isolated by cleaving the vector BluescriptKS-CTI-CD80-M79scFv (VL/VH) with EcoRI and SalI (Boehringer Mannheim) and subsequently introduced into the eukaryontic expression vector pEF-DHFR described in Mack et.al., Proc.Natl.Acad.Sci.U.S.A. 92 (1995), 7021-7025. containing the dihydrofolatereductase gene as selection marker.
For the final step of constructing the CD80-Ckappa chain, the Ckappa fragment obtained as described above was cleaved with the restriction enzymes BspEI and SalI and cloned into the vector pEF-DHFR-CTI-M79scFv-CD80 using the same enzymes. Thereby the M79scFv fragment was replaced by Ckappa. The final plasmid pEF-DHFR-CTI-CD80-Ckappa shown in figure 3 was linearized with the

restriction enzyme NdeI (Boehringer Mannheim) and transfected into CHO cells by electroporation. The electroporation conditions were 260V/960µF using a BioRad Gene Pulser™. Stable expression was performed in DHFR deficient CHO-cells as described by Kaufmann R.J. et.al. (1990) Methods Enzymol. 185, 537-566. The cells were grown for selection in nucleoside free α-MEM medium supplemented with 10% dialyzed FCS, 2 mM L-glutamine, 100U/ml Penicillin and 100ng/ml Streptomycin.

Example 1.1.2 Construction of the M79scFv-CH1 chain

In the next step the M79scFv-CH1 chain was assembled. The CH1 fragment of the human IgG1 heavy chain was amplified by PCR from the same cDNA template used for PCR-amplification of the human Ckappa-domain. The 5' PCR-primer introduced two cleavage sites (BspE1 and Nhe1) as well as the upper Hinge region of human IgG3 (5'CH1huG1BspE1: AAT TCC GGA ACC CCG CTG GGT GAC ACC ACC CAC ACC GCT AGC ACC AAG GGC CCA TCG GTC TTC C). The 3' PCR primer introduced the cleavage sites BspE1 and Spe1 (3'CH1huG1BspE1: AAT TCC GGA ACT AGT TTT GTC ACA AGA TTT GG). The resulting PCR-fragment was prepared for cloning by cleavage with the restriction enzyme BspE1 and inserted into the above described BS-CTI vector which was cleaved with BspE1 and Xma1. In order to avoid vector self ligation, the vector was treated with alkaline phosphatase. Thereafter, the CH1 fragment was excised from BS-CTI with the restriction enzymes Sal1 and BspE1 in order to connect it with the M79 single chain Fv-fragment as described below:

The M79 antibody was described by Göttlinger et.al.(1986) Int.J.Cancer:38, 47-53. The M79 scFv fragment was obtained from the bispecific single-chain antibody (M79scFv-antiCD3scFv) described by Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025. The DNA-fragment encoding this bispecific single-chain antibody was excised from the expression vector pEF-DHFR and inserted into the expression vector pEF-ADA by using the restriction enzymes EcoRI and SalI respectively The expression vector pEF-ADA was derived from the expression vector pEF-DHFR (Mack et.al. Proc.Natl.Acad.Sci. U.S.A.92 (1995) 7021-7025) by replacing the cDNA encoding murine dihydrofolate reductase (DHFR) by that encoding murine deaminase.

In order to introduce the CH1 fragment and thereby replacing the anti-CD3scFv fragment, the vector pEF-ADA containing the bispecific single-chain antibody M79scFv-antiCD3scFv was cleaved using the same restriction enzymes as for preparation of the CH1 fragment (BspE1,Sal1). The resulting plasmid pEF-ADA-M79scFv-CH1 shown in figure 3 was linearized with NdeI and transfected into CHO cells already transfected with the expression the vector pEF-DHFR-CTI-CD80-Ck. The double transfected cells were grown for selection in nucleoside free α-MEN-medium supplemented with 10% dialyzed FCS and 2 mM L-glutamine, 100 U/ml Penicillin, 100ng/ml Streptomycin, 0,1μM deoxycoformycin (dCF) and 1x1.1-AAU-additive as described by Kaufmann-RJ (Meth.Enzym.185 (1990) 537-566). After cells were successfully grown under these conditions the concentration of dCF was increased to 0,3μM (ADA selection) and MTX was added to a final concentration of 20nM (DHFR selection) in order to obtain higher expression levels of the Heterominbody due to gene amplification. ELISA-analysis of the culture supernatant of the transfected cell lines was carried out in order to determine the expression level of the Heterominibody and to confirm its binding specificity for the 17-1A antigen (see example 4.4 and figures 9, 10)

Example 1.2.1 M79scFvCK/CD80CH1 Heterominibody

Another version of the CD80 Heterominibody was constructed by replacing the M79scFv and the CD80 fragment by each other. Therefor the two expression plasmids pEF-DHFR-CTI-CD80-Ck and pEF-ADA M79scFv-CH1 were cleaved with EcoR1 and BspE1. The M79scFv fragment was then ligated with the pEF-DHFR-CTI-CK fragment and the CD80 fragment was ligated with the pEF-ADA-CH1-fragment. First, the vector pEF-DHFR-M79scFv-CK was transfected into CHO cells and grown for selection as described in example 1.1. The pEF-ADA-CD80-CH1 was transfected into the same CHO-cells in a second step and the resulting double transfected CHO cells were grown for selection as described above (see figure1 and 3).

1.2.2 Amplification, Subcloning and Purification of th M79scFvCK/CD80CH1 Heterominibody

Primary selection was carried out in nucleoside-free alpha MEM culture medium supplemented with 10% dialyzed FCS and 0,1µM deoxycoformycin (dCF) and 1x1.1-AAU-additive as described (Kaufman, Methods Enzymol. 185 (1990), 537-566). The expression of this construct was increased by gene amplification induced by stepwise increasing the concentrations of the DHFR-inhibitor methotrexate (MTX) and of the ADA-inhibitor deoxyformycin dCF.

The single amplification steps were carried out as follows:

1. Amplification 20nM MTX and 0,3µM dCF,
2. Amplification 100µM MTX and 1µM dCF
3. Amplification 500nM MTX and 3µM dCF. The cells obtained from the third amplification step were cloned by limiting dilution. Therefor the cells were seeded at a concentration of 50 cells per ml, 10 cells per ml and 2 cells per ml into 96 well flat-bottom tissue culture plates according to Current Protocols in Immunology (Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992) under the culture conditions of the third amplification step. Positive clones from wells with single tight cluster of cells as an evidence for monoclonal growth were identified by ELISA as described in Example 2.1.

One positive clone was expanded and taken for protein production. Large scale antibody production was carried out in rollerbottles using 500 ml medium.

The M79scFvCK/CD80CH1 Heterominibody was purified via its C-terminal histidine tail as described. (Mack et.al. Proc.Natl.Acad.Sci. U.S.A.92 (1995). 7021-7025).

Example 1.2..3 ELISA on cell-culture supernatant of CD80 Heterominibodies

To analyze the 17-1A binding properties of both CD80 Heterominibody versions and to confirm the proper association of CH1 and CK two different ELISAs were carried out. Specific binding to the 17-1A-antigen was shown by incubation of culture supernatant on immobilized recombinant 17-1A-antigen and detection of bound CD80-Heterominibodies by an anti-CD80-antibody. The heterodimeric structure of the Heterominibodies was confirmed by incubation of the culture supernatant on immobilized anti-His-tag-antibody followed by a detection step with an anti–human C_{kappa}-antibody. For details about both ELISAs see example 4.4.,figure 9 and 10.

Example 2: Analysis of Heterominibodies M79scFvCK/CD80CH1 and M79scFvCH1/CD80CK

2.1 ELISA-analysis of Heterominibody M79scFvCK/CD80CH1 expressed by transfected CHO-cell lines at different steps of gene amplification

The culture supernatants corresponding to primary selection, first and second amplification and third amplification plus subsequent cell-cloning were tested by ELISA. For this purpose recombinant 17-1A (Mack, Proc. Natl. Acad. Sci. USA 92 (1995) 7021-7025) was coated to 96 well U-bottom ELISA plates (Nunc maxisorb) (50µg/ml, 50µl/well) in phosphate buffered saline (PBS). Coating was performed overnight at 4°C, blocking was performed with 3% bovine serum albumin (BSA) in PBS for one hour at room temperature. Antibody constructs as culture supernatants from primary selection (PS) and from different amplification steps (1.Amp, 2.Amp, 3.Amp subcloned) (figure 4), respectively, were added and incubated for one hour at room temperature. Bound Heterominibody was detected by a biotin labeled anti-human CK antibody (Pierce, Cat No.31780) diluted 1:1000 in PBS 1%BSA. After three times of washing with PBS 0,05% Tween20, Avidin Peroxidase (DAKO,Hamburg, Cat.no P0347) diluted 1:1000 was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the following substrate solution: 22 mg ABTS (2,2 Azino-bis (3-Ethylbenzthiazoline-6 Sulfonic Acid) Diammonium salt) was dissolved in 10 ml 0,1M citrat buffer pH 5,1 containing 2,3mg Sodium perborate Tetrahydrate. For negative controls, the plates were incubated with PBS instead of bifunctional antibody constructs. The colored precipitate was measured at 405 nm using an ELISA-reader. As shown in figure 4, Heterominibody expression continuously increased from primary selection to a cell clone from third amplification step.

2.2 ELISA–analysis of Heterominibodies M79scFvCK/CD80CH1 and M79scFvCH1/CD80CK by different combinations of immobilized antibodies or immobilized 17-1A-antigen with appropriate detection antibodies

2.2.1.ELISA -analysis with immobilized 17-1A-antigen and an anti-CD80 detection antibody

The culture supernatants derived from the 1.Amplification step were tested. Recombinant 17-1A-antigen was coated to the ELISA plate. Bound antibody constructs were detected by a CD80-specific monoclonal antibody (Immunotech, Cat. No. 1449) diluted 1: 1000 in PBS 1% BSA followed by a polyclonal peroxidase-conjugated goat anti-mouse IgG-antibody (Fc-specific) diluted 1: 1000 in PBS 1% BSA. The ELISA procedure was performed as described above. As shown in figure 9 both Heterominibody versions proved to bind to the 17-1A antigen although the M79scFvCK/CD80CH1-version showed a substantially higher expression level than the M79scFvCH1/CD80CK-version.

2.2.2 ELISA –analysis with immobilized anti-His-tag-antibody and an anti-human C_{kappa} detection antibody

The culture supernatants derived from the 1.Amplification step were tested. BSA-free anti-histidine tag-antibody (Dianova, Hamburg,Cat No DIA910) was coated to the ELISA plate. Bound antibody constructs were detected by a biotin labeled anti-human C_{kappa} antibody (Pierce, Cat No.31780) diluted 1:1000 in PBS followed by Avidin Peroxidase (DAKO, Hamburg, Cat. No P0347) diluted 1:1000 1%BSA. The ELISA procedure was performed as described above. As shown in figure 10 the results of this ELISA confirm the higher expression level of Heterominibody M79scFvCK/CD80CH1 and together with the results of the foregoing ELISA clearly demonstrate the heterodimeric structure of the CD80-Heterominibodies.

Example 2.3. SDS-Proteingel

To determine the size of the purified Heterominibody M79scFvCK/CD80CH1 SDS-PAGE was carried out with a 10% polyacrylamid-gel under nonreducing conditions according to Laemmli (Laemmli, Nature 227 (1970), no. 259 680-5) gel followed by protein staining with ROTI®-Blue (Carl Roth GmbH + Co, Karlsruhe, Germany; Cat No A152.1). Compared to the molecular standard used (Rainbow™ colored protein

molecular weight marker, range 14.300-2.200.000, Amersham LIFESCIENCE, Braunschweig, Germany, Cat No RPN 756) a distinct protein band could be seen at about 115kD which is in accordance with the expected molecular.

Example 2.4 Flowcytometric analysis of Heterominibody M79scFvCK/CD80CH1

The binding of Heterominibody M79scFvCK/CD80CH1 to native 17-1A-antigen was analyzed by flowcytometry. 200.000 17-1A transfected CHO cells (see example 5.1) were incubated for 30 minutes with several dilutions of purified Heterominibody ranging from 4µg/ml to 3,9ng/ml. Thereafter, cells were washed twice in PBS and incubated for another 30 minutes with a R-Phycoerythrin conjugated murine anti-human CD80 antibody (Becton Dickinson Immunocytometry Systems, San Jose CA, USA, Cat.no. 340294). After two final washing steps in PBS, cells were analyzed by flowcytrometry (FACS-SCAN Becton Dickinson Immunocytometry Systems, San Jose CA, USA) For results see figure 5.

Example 3: CD80-antiLeyscFv-Heterominibodies constructs

3.1 Heterominibody antiLeyscFvCH1/CD80CK construct

A CD80-Heterominibody with another antigen specificity (anti-LewisY(LeY) was constructed by replacing the 17-1A-specific antigen binding-region M79scFv by the scFv-fragment of a murine monoclonal antibody directed against the LewisY-antigen (LeY), expressed on many epithelial tumor cells. For construction strategy see figure 1 and 3.For generating the antiLeyscFv-CH1-chain the vector pEF-ADA-M79scFv-CH1 described in example 1.1.2 and shown in figure 3 was cleaved with the restriction enzymes EcoRI and BspEI thus releasing the M79scFv-fragment including the N-terminal eukaryotic leader sequence. This scFv-fragment was then replaced by that of the LeY-specific antibody also carrying a eukaryotic leader at its N-terminus. The sequence of the corresponding EcoRI/BspEI-DNA-fragment is shown in figure 6. Subcloning was carried out in the E.coli strain XL-1 blue following standard methods (Sambrook, Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, NY (1989). The resulting expression plasmid pEF-ADA-anti LeYscFv-CH1 was

linearized with NdeI and transfected into CHO-cells that were already transfected with the expression plasmid pEF-DHFR-CTI-CD80-Ckappa described in example 1 and shown in Figure 7.Primary selection of the resulting double transfectants was carried out as described in example 1. The expression of the Heterominibody anti-LeYscFvCH1/CD80CK was subsequently increased by gene amplification induced by the addition of the DHFR-inhibitor methotrexate (MTX) to a final concentration of 20nM, and the increase of deoxycoformycin (dCF) to a final concentration of 0,3μM described (Kaufman, Methods Enzymol. 185 (1990), 537-566).

3.2 Heterominibody antiLeyscFvCK/CD80CH1

For construction of Heterominibody antiLeYscFvCK/CD80CH1 the M79scFv-fragment was excised from the vector pEF-DHFR-M79scFv-CK described in example 1 using the restriction enzymes EcoRI and BspEI and replaced by the EcoRI/BspEI fragment of the antigen binding region of the LeY-specific antibody as shown in figure 6. Subcloning was carried out in the E.coli strain XL-1 blue following standard methods (Sambrook, Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, Cold Spring Habour, NY (1989)).

The resulting expression plasmid pEF-DHFR-anti LeYscFv-CK was transfected into CHO-cells followed by transfection of the expression plasmid pEF-ADA-CD80-CH1 described in example 1. In order to increase the expression of Heterominibody anti-LeYscFv-CK/CD80CH1, gene amplification was carried out as described above for Heterominibody antiLeYscFvCH1/CD80CK.

3.3.1 ELISA-analysis of Heterominibody antiLeyscFvCK/CD80CH1 and Heterominibody antiLeyscFvCH 1/CD80CK with immobilized LeY-BSA-conjugate and an anti CD80 detection antibody

The culture supernatants of the corresponding transfectants harvested after the first step of gene amplification were tested by ELISA. For this purpose a commercially available BSA-conjugate of synthetic Lewis Y-antigen (Alberta Research Council, Canada) was coated to 96 well U-bottom ELISA plates (Nunc maxisorb) (30μg/ml 50μl/well) in phosphate buffered saline (PBS). Coating was performed over night at

4°C, blocking was performed with 3% bovine serum albumin (BSA) in PBS for one hour at room temperature. Culture supernatants from the first amplification step (1. Amp.) (figure 7), were added and incubated for one hour at room temperature at different dilutions made in PBS containing 1% BSA.

Bound Heterominibodies were detected by a CD80-specific monoclonal antibody (Immunotech, Cat No. 1449) diluted 1:1000 in PBS 1%BSA. After three times of washing with PBS 0,05% Tween20, a polyclonal peroxidase-conjugated Goat Anti-Mouse IgG-antibody (Fc-specific) diluted 1:5000 was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the following substrate solution: 22 mg ABTS (2,2 Azino-bis (3-Ethylbenzthiazoline-6 Sulfonic Acid) Diammonium salt) dissolved in 10 ml 0,1M citrat buffer pH 5,1 containing 2,3 mg Sodium perborate Tetrahydrate. For negative controls, the plates were incubated with PBS instead of culture supernatants. The coloured precipitate was measured at 405 nm using an ELISA-reader. The results are shown in figure 7 demonstrating the specifity of both Heterominibody versions for the LeY-antigen and their heterodimeric structur.

3.3.2 ELISA-analysis of Heterominibody antiLeyscFvCK/CD80CH1 and Heterominibody antiLeyscFvCH1/CD80CK with immobilized anti-His-tag-antibody and an anti-human C_{kappa} detection antibody of the corresponding transfectants harvested after the first step of gene amplification constructs using his-coating

The culture supernatants were tested by ELISA. For this purpose, a BSA-free anti-His-tag-antibody (Dianova, Cat No 910) was coated to 96 well U-bottom ELISA plates (Nunc maxisorb) (25µg/ml 50µl/well) in phosphate buffered saline (PBS). Coating was performed over night at 4°C, blocking was performed with 3% bovine serum albumin (BSA) in PBS for one hour at room temperature. Culture supernatants from the first amplification step (1. Amp.) (figure 8), were added and incubated for one hour at room temperature at different dilutions made in PBS containing 1% BSA.

Bound Heterominibodies were detected by a biotin labeled anti-human C_{kappa} antibody (Pierce, Cat No.31780) diluted 1:1000 in PBS 1%BSA. After three times of washing with PBS 0,05% Tween20, peroxidase-conjugated avidin (DAKO,Hamburg Cat No P0347) was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the following substrate solution: 22 mg ABTS (2,2 Azino-bis (3-Ethylbenzthiazoline-6 Sulfonic Acid) Diammonium salt) was dissolved in 10 ml 0,1M citrat buffer pH 5,1 containing 2,3 mg Sodium perborate Tetrahydrate. For negative controls, the plates were incubated with PBS instead of culture supernatant. The coloured precipitate was measured at 405 nm using an ELISA-reader. The results are shown in figure 8 confirming the results of the foregoing ELISA.

Example 4 Construction of M79scFv Heterominibodies with different costimulatory proteins (CD54, CD58, CD86)

Heterominibodies containing three further costimulatory (CD86) or adhesion proteins (CD54, CD58) were constructed. CD54, CD58, CD86 were introduced into both Heterominibody versions (see Example 1.1 and 1.2). The CD54, CD58 and CD86 fragments were obtained by polymerase chain reaction (PCR) using specific oligonucleotide primers complementary to the 5´ and 3´ ends of the nucleotide sequence encoding the extracellular part of these proteins. The cDNA template used for these PCRs were prepared by reverse transcription of the total RNA prepared from different cell lines as mentioned below according to standard procedures (Sambrook,Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, cold Spring Habour, New York (1989)).

4.1 Construction of two M79scFv-CD54 Heterominibody versions

The CD54 Heterominibodies were constructed by replacing the extracellular part of CD80 within the Heterominibodies described in example 1 by that of CD54.(figure 1). The construction strategy is described below.

4.1.1 Construction of the Heterominibody M79scFvCH1/CD54CK

The CD54 antigen known as ICAM-1 (Intercellular adhesion molecule-1) belongs to the Ig-superfamily. It is a heavily glycosylated protein which is expressed on many lymphoid cells. e.g. dendritic cells. A more detailed description was published by Simmons D. et.al. Nature 331 (1987) 624-626. The cDNA template was obtained by reverse transcription of the total RNA from TPA-stimulated HL-60-cells. To amplify the extracellular domain of CD54, specific primers for the 5´and 3´end were used. These primers also introduced the restriction cleavage-sites EcoR1 and BspE1 (5´ ICAM: CTC GAA TTC ACT ATG GCT CCC AGC AGC CCC CG and 3´ICAM: GAT TCC GGA CTC ATA CCG GGG GGA GAG CAC). The further cloning and expression procedure was the same as described in example 1.1. for the correspoding CD80-Heterominibody resulting in double transfected CHO cells (pEF-ADA M79scFvCH1, pEF-DHFR-CTI-CD54-CK; figure 3) that proved to secrete the CD54-Heterominibody into the cell-culture medium.

4.1.2 Construction of the Heterominibody M79scFvCK/CD54CH1

Another version of the CD54 Heterominibody was constructed by replacing the M79scFv-and the CD54-fragment by each other. For this purpose the two expression plasmids pEF-DHFR-CTI-CD54-CK and pEF-ADA-M79scFv-CH1 described above were cleaved with Nde1 and BspE1, respectively. The CD54 containing fragment was cloned into the pEF-ADA vector fragment containing CH1. The resulting expression plasmid pEF-ADA-CD54-CH1 (figure 3) was transfected into transfected CHO cells (see example 1.2.1) already transfected with the pEF-DHFR-M79scFv-CK (figure 3) as described in example 1.1.1. The double transfected CHO cells were grown for selection as described in example 1

Example 4.1.3 ELISA on cell-culture supernatants of M79scFv-CD54 Heterominibodies

To analyze the 17-1A-binding specifity of both CD54 Heterominibody versions and to confirm the the proper association of CH1 and CK two different ELISA were carried. Specific binding to the 17-1A-antigen and the heterodimeric structure of the Heterominibodies was shown by incubation of culture supernatant on immobilized recombinant 17-1A-antigen and detection of bound CD54-Heterominibodies by an anti-CD54 antibody. The heterodimeric structure of the Heterominibodies was

further confirmed by incubation of the culture supernatant on immobilized anti-His-tag-antibody followed by a detection step with an anti-human C_{kappa} antibody.For details about both ELISAs see example 4.4, figure 9 and 10.

4.2 Construction of two M79scFv-CD58 Heterominibody versions

The CD58 Heterominibodies were constructed replacing the extracellular part of CD80 within the Heterominibodies described in example 1 by that of CD58 (figure 1). The construction strategy is described below.

4.2.1 Construction of the M79scFvCH1/CD58CK Heterominibody

CD58 also known as LFA-3 (Lymphocyte Function-Associated Antigen) is a protein belonging to the Ig-superfamily and is the counterreceptor of CD2. A more detailed description was published by Wallner B.P. et.al. J.Exp.Med 166 (1987) 923-932). The cDNA template was obtained by reverse transcription of the total RNA from U937 cells. To amplify the extracellular domain of CD58 and to introduce the restriction enzyme cleavage sites Xba1 and BspE1, specific 5´and 3´primers were used (5`LFA-3 AA TCT AGA ACC ATG GTT GCT GGG AGC GAC G and 3´LFA-3 AAG TCC GGA TCT GTG TCT TGA ATG ACC GCT GC). The further cloning and expression procedure was the same as described in example 1 except that XbaI instead of EcoRI was used due to an internal EcoRI-site within the CD58-DNA-fragment and a dam-methylase deficient E.coli-strain was used in order to prevent blocking of the BspEI site at the 3´-end of the CD58-fragment due to an overlapping dam-site. The finally resulting double transfected CHO cells (pEF-ADA-M79svFv-CH1, pEF-DHFR-CTI-CD58-CK, see figure 3) proved to secrete CD58 Heterominibody into the cell-culture medium.

4.2.2 Construction of the Heterominibody M79scFvCK/CD58CH1

Another version of the CD58 Heterominibody was constructed replacing the M79scFv-and the CD58-fragment by each other (see figure 1). For this purpose two expression plasmids pEF-DHFR-CTI-CD58-Ck and pEF-ADA-M79scFv-CH1 described above were cleaved with Nde1 and BspE1, respectively. The CD58 containing fragment was cloned into the pEF-ADA vector fragment containing CH1.

The resulting expression plasmid pEF-ADA-CD58-CH1 (figure 3) was transfected into CHO-cells already transfected with pEF-DHFR-M79scFv-CK as described in example 1. The double transfected CHO cells were grown for selection as described in example 1

Example 4.2.3 ELISA on cell-culture supernatant of anti M79scFv-CD58-Heterominibodies

To analyze the 17-1A binding specificity of both CD58 Heterominibody versions and to confirm the proper association of CH1 and CK, two different ELISAs were carried out. Specific binding to the 17-1A-antigen and the heterodimeric structure of the Heterominibodies was shown by incubation of culture supernatant on immobilized recombinant 17-1A-antigen and detection of bound CD58-Heterominibodies by an anti-CD58-antibody. The heterodimeric structure of the Heterominibodies was further confirmed by incubation of the culture supernatant on immobilized anti-His-tag-antibody followed by a detection step with an anti-human C_{kappa} antibody. For details about both ELISAs see example 4.4

4.3 Construction of two M79scFv-CD86 Heterominibody versions

The CD86 Heterominibodies were constructed by replacing the extracellular part of CD80 within the Heterominibodies described in example 1 by that of CD86 (figure 1). The construction strategy is described below.

4.3.1 Construction of the M79scFvCH1/CD86CK Heterominibody

The CD86 costimulatory protein also known as B7-2 belongs to the Ig superfamily. It is a heavily glycosylated protein of 306 amino acids. A more detailed description was published by Freeman G.J.et.al. Science 262 (1993) 909-911. The cDNA template was obtained by reverse transcription of the total RNA from the Burkitt-Lymphoma cell line Raji. To amplify the extracellular domain of CD86 specific 5'and 3'primers (5`B7-2: 5'AAG TCT AGA AAA TGG ATC CCC CAG TGC ACT ATG3', 3`B7-2: 5'AAT TCC GGA TGG GGG AGG CTG AGG GTC CTC AAG C3') were used. These primers also introduce Xba1 and BspE1 cleavage sites which were used to clone the CD86 PCR-fragment into the vector Bluescript KS-CTI-

M79scFv. The further cloning and expression procedure was the same as described in example 1 except that XbaI instead of EcoRI was used due to an internal EcoRI-site within the CD86-DNA-fragment. The finally resulting double transfected CHO-cells (pEF-ADA-M79scFv-CH1, pEF-DHFR-CTI-CD86-CK) proved to secrete CD86-Heterominibody into the cell-culture medium.

4.3.2 Construction of the M79scFvCK/CD86CH1 Heterominibody

Another version of the CD86 Heterominibody was constructed by replacing the M79scFv-and the CD86-fragment by each other (figure 1). For this purpose the two expression plasmids pEF-DHFR-CTI-CD86-CK and pEF-ADA-M79scFv-CH1) were cleaved with Nde1 and BspE1, respectively. The CD86 containing fragment was cloned into the pEF-ADA vector fragment containing CH1. The resulting expression plasmid pEF-ADA-CD86-CH1 (figure 3) was transfected into CHO-cells already transfected with pEF-DHFR-M79scFv-CK as described in example 1. The double transfected CHO cells (pEF-DHFR-M79scFv-CK, pEF-ADA-CD86-CH1, figure 3) cells were grown for selection as described in example 1.

Example 4.3.3 ELISA on cell-culture supernatant of CD86 Heterominibody

To analyze the 17-1A binding specificity of CD86 Heterominibody versions and to confirm the proper association of CH1 and CK two different ELISAs were carried out. Specific binding to the 17-1A-antigen and the heterodimeric structure of the Heterominibodies was shown by incubation of culture supernatant on immobilized recombinant 17-1A-antigen and detection of bound CD86-Heterominibodies by an anti-CD86 antibody. The heterodimeric structure of the Heterominibodies was further confirmed by incubation of the culture supernatant on immobilized anti-His-tag antibody followed by a detection step with an anti-human C_{kappa} antibody. For details about both ELISAs see example 4.4 see figure 9 and 10.

Example 4.4 ELISA on cell-culture supernatants of M79scFv-Heterominibodyies

Two different ELISAs on cell-culture supernatants were performed for each M79scFv-Heterominibody:

Binding to the 17-1A-antigen was analyzed using recombinant 17-1A-antigen obtained by stable expression in CHO-cells as described (Mack et.al.

Proc.Natl.Acad.Sci. 92 (1995) 7021-7025). The recombinant antigen consists of the first 264 amino acids of the native 17-1A antigen also known as GA 733-2 (Scala, Proc.Natl.Acad.Sci. 87 (1990), 3542-3546) followed by a stop codon.. The antigen was immobilized on 96 well U bottom ELISA plates (nunc maxisorb) at a concentration of 50µg/ml phosphate buffered saline PBS. Coating was carried out at 4°C for 12 hours with 50µl followed by washing once with (PBS) 0,05%Tween. The ELISA was then blocked for 1 hour with PBS/3%bovine serum albumin (BSA) and washed again once. Subsequently, the cell-culture supernatant was added undiluted and at several dilutions and incubated for 2 hours. Specific detection was dependent on the type of costimulatory proteins associated with the different Heterominibody version. For specific antibodies and working dilutions see table 1. After three times of washing with PBS 0,05% Tween20, a polyclonal peroxidase-conjugated goat anti-mouse IgG-antibody (Fc-specific) was added and incubated at room temperature for one hour. After four times of washing with PBS 0,05% Tween20, the ELISA was finally developed by adding the following substrate solution: 22 mg ABTS (2,2 Azino-bis (3-Ethylbenzthiazoline-6 Sulfonic Acid) Diammonium salt) dissolved in 10 ml 0,1M citrate buffer pH 5,1 containing 2,3 mg Sodium perborate Tetrahydrate. For negative controls, the plates were incubated with PBS instead of culture supernatants. The colored precipitate was measured at 405 nm using an ELISA-reader (figure 9). The results shown in figure 9 clearly demonstrate that each of the constructed M79scFv-Heterominibodies could be detected as fully functional heterodimer in the supernatant of the corresponding transfectants.

For the second ELISA an anti-His-tag-antibody (DIANOVA; Hamburg Cat.no. DIA 910) diluted 1:40 was coated to 96-well plates as described above. Supernatants of all Heterominibody versions were added pure and in several dilutions. A biotinylated anti human Ckappa antibody followed by peroxidase-conjugated streptavidin (1:1000) (DAKO, Hamburg Cat.no P0347) was used for detection of bound Heterominibodies (see table 1). The ELISA was developed as described above. For results see figure 10.

Example 5 Stimulation of naive CD4+CD45RO- T cells by M79scFcCK/CD80CH1 Heterominibody

Example 5.1 Purification of naive CD4+CD45RO- from th peripheral blood of healthy human donors blood

To analyze the biological function of the M79scFvCK/CD80CH1- Heterominibody, a CD4+ T-cell stimulation experiment was performed. CD4+CD45RO- T-cells, commonly considered to be naive, were isolated from of peripheral blood of healthy donors by negative selection. First, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Density Gradient (Current Protocols of Immunology, Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992). After washing the cells three times with phosphate buffered saline (PBS) supplemented with 2% fetal calf serum (FCS), CD4+ T-cells were purified by using commercially available CD4+-T-cell columns (R&D Systems,Minneapolis MN USA, Cat no HCD43). In the next step CD45RO+ T-cells were removed by paramagnetic Dynabeads M450 (Dynal, Hamburg, Cat.No. 110.02). For this purpose, CD4+ T-cells were incubated for 30 minutes with the murine anti-human CD45RO antibody (UHCL-1) at a concentration of 10µg/ml. Subsequently the cells were washed twice and thereafter incubated for another 30 minutes with magnetic beads conjugated with the sheep anti-mouse IgG1 antibody M450. The CD4+CD45RO+ T-cells that were quantitatively attached to magnetic beads were then removed by the application of magnet. The remaining cells were CD4+ and CD45RO- with a purity of 98% as confirmed by flowcytometry.

Example 5.2 Stimulation of naive CD4+CD45RO- T-cells by simultaneous incubation with the M79scFvCK/CD80CH1 Heterominibody and/or bispecific single chain antibody M79scFv-antiCD3scFv

CD4+CD45RO- T-cells were purified as described above. The stimulation was performed in 96-well TPP flat-bottom plates. The stimulation assay was carried out as follows. 17-1A transfected CHO-cells were used as stimulator cells. This 17-1A transfected cell-line was generated by subcloning of a DNA-fragment encoding the complete amino acid sequence of the 17-1A-antigen also known as GA733-2 (Szala, Proc. Natl. Acad. Sci. USA 87(1990) 3542-3546), into the eukaryotic expression vector pEFDHFR according to standard procedures (Sambrook, Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour

Laboratory Press, Cold Spring Habour, NY (1989); linearization of the resulting plasmid with the restriction enzyme Nde I and subsequent stable transfection into DHFR-deficient CHO cells was performed as described in example 1.1.1. The expression of transmembrane 17-1A was increased by gene amplification induced by stepwise addition of increasing concentrations of the DHFR-inhibitor Methotrexat (MTX) to a final concentration of 500nM, with the concentration steps in between being 20nM and 100nM (Kaufman, Methods Enzymol. 185 (1990), 537-566).

These cells were tested for membrane expression of 17-1A by flowcytometry using the 17-1A-specific monoclonal antibody M79 (Göttlinger, Int. J. Cancer 38 (1986) 47-53) at a concentration of 10 μg/ml followed by a polyclonal goat anti mouse IgG + IgM (H+L) antibody diluted 1:100 in PBS. As negative control untransfected CHO cells were used whereas the 17-1A-positive human gastric cancer cell-line Kato, obtained from ATCC served as positive control. Results are shown in figure 11.

Before using these cells T-cell stimulation they were irradiated with 14000 rad, washed twice in PBS, 2%FCS, counted, diluted in medium (for details see below) and seeded into 96-well plates at a number of 25.000 cells per well. 50.000 CD4+CD45RO-T-cells cells were added to each well thus resulting in a T-cell/stimulator cell ratio of 2:1. M79scFvCK7CD80CH1- Heterominibody and/or the bispecific single chain antibody M79scFv-antiCD3scFv were added in several concentrations as shown in (Table 2) Cells were grown in RPMI 1640 medium supplemented with 10% human AB serum, 100U/ml Penicillin, 100mg/ml Streptomycin, 2mM Glutamin, 1mM sodium pyruvat, 10mM HEPES-buffer, 50μM Mercaptoethanol at 37°C 6% CO2 and 100% humidity for up to 12 days.

5.3 BrdU-Proliferationassay

In order to measure the proliferation kinetics of CD4+CD45R0- T-cells simultaneously stimulated by M79scFvCK/CD80CH1-Heterominibody and the bispecific single chain antibody M79scFv-antiCD3scFv or stimulated with the bispecific single chain antibody alone, a BrdU proliferation assay was performed. For details see product description by Boehringer Mannheim Cat.No. 1647229. The results shown in figure 12 clearly demonstrate a substantially increased cell proliferation induced by Heterominibody plus the bispecific single chain antibody compared to that induced by the bispecific antibody alone.

5.4 Flowcytometric analysis of CD4+CD45R0- T-cells stimulated with the M79scFvCK/CD80CH1 Heterominibody and/or the bispecific single chain antibody M79scFv-antiCD3scFv

CD45RO and CD45RA expression levels on stimulated T-cells were analyzed by flowcytometry (FACS Scan, Becton Dickinson) on day 3 and 6 of the stimulation experiment. Stimulated T-cells as well as controls (see example 5.1) were incubated for 30 minutes with different combinations of antibodies listed in table 4.

T-cells were incubated with the following three antibody combinations: anti CD45RO FITC/ anti CD45RA PE, anti CD45RO FITC / Isotyp IgG1 PE, anti CD45RA PE/ Isotyp IgG2a FITC. The percentage of primed T-cells that switched to the surface phenotype CD45R0+/CD45RA- depending on the concentrations of Heterominibody and bispecific antibody is shown in figures 13 and 14 corresponding to a stimulation time of 3 and 6 days, respectively.

5.5 INF-γ ELISA analysis of cell culture supernatant of stimulated CD4+ T-cells

In order to confirm in vitro priming of CD4+ T-cells by the combination of Heterominibody and bispecific single chain antibody, the INF-γ-concentration in the T-cell culture supernatant was determined using a semi-quantitative INF-γ ELISA (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat No. 80-3932-00) was performed according to manufacture's manual.. Since INF-γ is typically secreted by primed TH1- but not by naive CD4+ T-cells, the results shown in figures 15 and 16 demonstrate that T-cell priming has occurred in the presence of both Heterominibody and bispecific single chain antibody but not with the bispecific antibody alone. Furthermore the secretion of INF-γ by the primed CD4+T-cells strongly indicates the differentiation of these cells into TH1-phenotype.

Example 5.6 IL-5 ELISA –analysis of cell culture supernatant of stimulated CD4+ T-cells

A second ELISA was performed analyzing the IL-5 secretion of stimulated CD4+ cells. However the IL-5 ELISA (Genzyme DuoSet, genzyme Diagnostics Cambridge, MA USA Cat.No. 80-5025-00) of T-cell culture supernatant did not detect any IL-5 secretion as shown in figures 17 and 18 Since IL-5 typically secreted is by primed CD4+ T-cells of the TH2-phenotype, the combined T-cell stimulation by the M79scFvCK/CD80CH1 Heterominibody and the bispecific single chain antibody M79scFv-antiCD3scFv proved to induce no priming of TH2 T-cells at all.

Example 6 Costimulation of CD8+CD45RO- T cells by theM79scFVCKCD80CH1-Heterominibody

Example 6.1 Purification of naive CD8+CD45RO- T-cells from the peripheral blood of healthy human donors

To analyze the biological function of the M79scFv-CK/CD80-CH1 Heterominibody, a CD8+ T-cell stimulation experiment was performed. CD8+CD45RO- T-cells, commonly considered to be naive, were isolated from peripheral blood of healthy donors by negative selection. At first, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Density Gradient (Current Protocols of Immunology, Coligan, Kruisbeek, Margulies, Shevach and Strober, Wiley-Interscience, 1992). After washing the cells three times with phosphate buffered saline (PBS) supplemented with 2% fetal calf serum (FCS), CD8+T-cells were purified, using commercially available CD8+-T-cell columns (R&D Systems,Minneapolis MN USA, Cat No HCD8C-1000). In addition to the manufacture´s protocol 1µg/ml murine anti human CD11b antibody was added to the supplied antibody cocktail in order to remove the suppressor T-cells that are CD11b+/CD25-. In the next step CD45RO+ T-cells were removed by use of paramagnetic Dynabeads M450 (Dynal, Hamburg, Cat.No. 110.02) For this purpose CD8+T-cells were incubated for 30 minutes with the murine anti-human CD45RO antibody (UHCL-1) at a concentration of 10µg/ml.

Subsequently, the cells were washed twice and thereafter incubated for another 30 minutes with magnetic beads conjugated with the sheep anti-mouse IgG1 antibody M450. The CD8+CD45RO+T-cells that were quantitatively attached to magnetic beads were then removed by the application of a magnet The remaining cells were CD8+CD45RO-CD28+, with a purity of 95-98%.

Example 6.2 Stimulation of naive CD8+CD45R0-T-cells by simultaneous incubation with the M79scFvCK/CD80CH1-Heterominibody and/or the bispecific single chain antibody M79scFv-anti CD3scFv

CD8+CD45RO- were stimulated as described in example 5using construct concentrations as displayed in table 3

BrdU Proliferationassay as well as FACS analysis were performed as described in example 5. For results see figures 19, 20, 21

Example 6.3 TNF-α ELISA analysis of cell culture supernatant of stimulated CD8-T-cells

In order to confirm in vitro priming of CD8+T-cells by the combination of Heterominibody and bispecific antibody, the TNF-α-concentration in the T-cell culture supernatant was determined using a semiquantitative TNF-α ELISA (Genzyme DuoSet, Genzyme Diagnostics Cambridge, MA USA Cat No. 80-3932-00) which was carried out according to manufacture´s manual. Since TNF-α is typically secreted by primed but not by naive CD8+T-cells the results shown in figure 22 demonstrate that T-cell priming has occured in the presence of both Hetrominibody and bispecific antibody but not with the bispecific antibody alone.

Example7 Heterominibody M79scFvCKantiCD3scFv/CD80CH1

Another version of the CD80 Heterominibody was constructed by adding an antiCD3scFv-fragment via a Glycin$_4$Serin$_1$-linker to the C-terminus of the M79scFvCK-polypeptide chain described in example 1. For this purpose, the DNA-fragment encoding this polypeptide chain was excised from the expression plasmid

pEF-DHFR-M79scFv-CK described in example 1 and subcloned in the vector pMa (Stanssens, Nucleic Acids Res.17(1998)441-4454) using the restriction enzymes EcoRI and SalI (Boehringer,Mannheim). The antiCD3scFv-fragment was PCR-amplified from the DNA template encoding the bispecific single chain antibody M79scFv-antiCD3scFv described by Mack, Proc.Natl.Sci.U.S.A. 92 (1995) 7021-7025 by using the following primers. The 5'primer VHTR66CKSAC (5'-CCT GAG CTC GCC CGT CAC AAA GAG CTT CAA CAG GGG AGA GTG TGG AGG TGG TGG ATC CGA TAT C-3'), introduced a SacI-site and the 3'primer VLTR66SalNotXba introduced the cleavage sites SalI , NotI and XbaI (5'-ATT CTA GAG CGG CCG CGT CGA CTA TTT CAG CTC CAG CTT GGT CCC AGC –3') The resulting PCR fragment was cloned according to standard procedures (Sambrook,Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, cold Spring Habour, New York (1989)) into the pMa-vector by using the restriction enzyme cleavage sites SacI and XbaI (Boehringer Mannheim). In the final step the whole M79scFvCKantiCD3scFv-fragment was excised from the pMa-vector by the restriction enzymes EcoRI and SalI and subcloned into the eukaryotic expression vector pEF-DHFR described by Mack, Proc.Natl.Acad.Sci. 92 (1995)7021-7025 The resulting expression plasmid was transfected into DHFR-deficient CHO-cells, prior to transfection with the expression-plasmid pEF-ADA-CD80-CH1 described in example 1. Double transfection and selection of the CHO-cells was carried out as described in example 1

Example 8: CD80-M79scFv constructs

8.1 CD80 - M79 scFv (VL/VH) construct with short $(Gly_4Ser_1)_1$ linker

A protein was constructed that consists of the single-chain Fv fragment(scFv) of the murine anti 17-1A antibody M79 and the extracellular part of the human costimulatory protein CD80 (B7-1) connected by a $(Gly_4Ser_1)_1$ linker (Figure24). The M79 antibody was obtained as described by Göttlinger et.al.(1986) Int.J.Cancer:38, 47-53. The M79 scFv fragment was cloned as described by Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025. The complete plasmid was cloned in several steps. First a poly-linker designated CTI was inserted into the Bluescript KS vector (GenBank® accession number X52327) using the restriction enzym cleavage

sites XbaI and SalI (Boehringer Mannheim). The introduction of the polylinker CTI provided additional cleavage sites as well as the sequence encoding the $(Gly_4Ser_1)_1$, linker a six-amino acid histidine tag and a stop codon as shown in Figure 2 .The vector Bluescript KS + CTI was prepared by cleavage with the restriction enzyms EcoRV and XmaI (Boehringer Mannheim and New England Biolabs) in order to ligate it (T4 DNA, Ligase Boehringer Mannheim) with the M79 scFv fragment cleaved by EcoRV and BspEI (). The resulting vector Bluescript KS+CTI+M79 scFv again was cleaved with EcoRI (Boehringer Mannheim) and BspEI in order to insert the CD80 DNA-fragment which was previously prepared using the same enzyms. Prior to subcloning, the CD80 fragment was obtained by polymerase chain reaction (PCR) using specific oligonucleotide primers complementary to the 5´ and 3´ ends of the nucleotide sequence encoding the extracellular part of CD80 (Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.). These primers also introduced an EcoRI and a BspEI cleavage site (5´CD80 Primer: 5´GCA GAA TTC ACC ATG GGC CAC ACA CGG AGG CAG 3´; 3´CD80 Primer: 5´TGG TCC GGA GTT ATC AGG AAA ATG CTC TTG CTT G 3´) The cDNA template used for this PCR was prepared by reverse transcription of the total RNA prepared from the Burkitt-lymphoma cell line Raji according to standard procedures (Sambrook,Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press, cold Spring Habour, New York (1989)).

The CD80 costimulatory protein belongs to the Ig superfamily. It is a heavily glycosylated protein of 262 amino acids. A more detailed description was published by Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722.

In the last step, the whole CD80-M79scFv (VL/VH)DNA fragment (figure 25.) was isolated by cleaving the vector Bluescript KS+CTI+CD80+M79scFv (VL/VH) with EcoRI and SalI (Boehringer Mannheim) and subsequently introduced into the eukaryontic expression vector pEF-DHFR described in Mack et.al. Proc.Natl.Sci.U.S.A. 92 (1995) 7021-7025. containing the dihydrofolatereductase gene as selection marker.The final plasmid was linearized with the restriction enzym NdeI (Boehringer Mannheim) and transfected into CHO cells by electroporation. The electroporation conditions were 260V/960µF using a BioRad Gene Pulser™. Stable expression was performed in DHFR deficient CHO-cells as

described by Kaufmann R.J.et.al. (1990) Methods Enzymol. 185, 537-566. The cells were grown for selection in nucleoside free α-MEM medium supplemented with 10% dialysed FCS and 2 mM L-glutamine. For production of the bifunctional CD80-M79 scFv (VL/VH)construct, cells were grown in rollerbottles (Falcon) for 7 days in 300ml culture medium. The protein was purified via its His-tag attached to the C-terminus (see figure24) by using a Ni-NTA-column (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025).To analyse the binding properties different ELISA were performed:

8.1.1 ELISA with cell culture supernatant using anti-His-tag detection

Binding to the 17-1A-antigen was analysed using soluble 17-1A-antigen obtained as described (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025) by stable expression in CHO-cells of the DNA encoding the first 264 amino acids of the 17-1A antigen also known as GA 733-2 (Scala, Proc.Natl.Acad.Sci. 87 (1990) 3542-3546) followed by a stop codon.. The antigen was immobilized on 96 well U bottom ELISA plates (nunc maxisorb) at a concentration of 50µg/ml phosphat buffered saline PBS. Coating was carried out at 4°C for 12 hours with 50µl followed by washing once with (PBS) 0,05%Tween. The ELISA was then blocked for 1 hour with PBS/3%bovine serum albumin (BSA) and washed again once. Now the cell-culture supernatant was added undiluted and at several dilutions and incubated for 2 hours. As detection system a murine IgG1 anti His-tag antibody (dianova, Hamburg) diluted 1:200 and a peroxidase conjugated polyclonal goat anti mouse IgG (Fc) (dianova,Hamburg) antibody were applied sequentially. The ELISA was developed by adding ABTS-substrate solution (2´2 Azino-bis (3-Ethlbenzthiazoline-6-Sulfonic Acid), SIGMA A-1888, Steinheim) as described in example 2.1 . The result was measured by an ELISA-Reader at OD 405 nm; results are shown in Figure 26. Obviously no binding activity could be measured. As negative controls, the plates were incubated with PBS instead of antibody constructs. As positiv control served the anti-17-1A/anti-CD3 bispecific-single-chain antibody described previously (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995) 7021-7025).

8.1.2 ELISA with cell culture supernatant using anti-CD80 detection

Immobilization of 17-1A-antigen, blocking and the incubation of cell culture supernatants was performed as described above. Detection was carried out with a murine IgG1 anti-CD80-antibody diluted 1:1000 (dianova,Hamburg) followed by a peroxidase conjugated polyclonal goat anti-mouse IgG (Fc)-antibody diluted 1:5000 (dianova,Hamburg). The ELISA was developed with ABTS-substrat solution and OD-values were measured as described above, however, again no 17-1A-binding activity could be detected. As positiv control, the anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack et.al. Proc.Natl.Acad.Sci. 92 (1995)7021-7025) was used and detected with the described anti-His-tag antibody. Results are shown in Figure 27.

8.1.3 ELISA-analysis of purified recombinant CD 80-M79scFv-construct

As the ELISAs with cell-culture supernatants detecting specific antigen binding were all negativ, soluble CD80-M79scFv was obtained by protein purification from supernatant of a roller bottle culture (300ml) in order to exclude the possibility that no recombinant protein was secreted into the supernantant.The purification was carried out using a Nickel-NTA-column as described (Mack,M et.al.Proc.Natl.Sci.U.S.A. 92 (1995) 7021-7025). ELISA wells were coated with the protein eluted from the Nickel-NTA-column. Detection of the bifunctional CD80-M79scFv-construct was performed independently of its 17-1A-antigen binding activity by using either an anti His-tag antibody (see example 8.1.1.) as well as an anti-CD80 antibody (see example 8.1.2.) in seperate experiments followed by an anti-mouse IgG(Fc) antibody, respectively. Development of the ELISA as well as the measurement of the OD-values was carried out as described above. The results are shown in Fig 28., confirming the presence of the CD80-M79scFv-construct in the cell culture supernatant.

8.2. CD80 - M79 scFv (VH/VL) construct with $(Gly_4Ser_1)_1$ linker

To change the arrangement of the Ig variable regions within the M79scFv fragment from VL/VH to VH/VL a two step fusion PCR using oligonucleotide primers 5´VHB5RRV:AGG TGT ACA CTC CGA TAT C(A,C)A (A,G)CT GCA G(G,C)A GTC (A;T)GG, 3´VHGS15: 5´GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA G 3´, 5´VLGS15: 5´GGC

GGC GGC GGC TCC GGT GGT GGT GGT TCT GAC ATT CAG CTG ACC CAG TCT CCA3´ and 3´VLBspEI: 5´AAT CCG GAT TTG ATC TCG AGC TTG GTC CC3´ was performed according to the procedure described by Mack et al.Proc.Natl.Acad.Sci. 92 (1995) 7021-7025 (see also example 2.1.) The PCR-fragment encoding the VH/VL-scFv-fragment was cleaved with the restriction enzyms EcoRV/BspEI and inserted into the vector Bluescript KS + CTI already prepared by cleavage with EcoRV/XmaI (see example 8.1.).Next, the inverted M79scFv (VH/VL) fragment was excised with the restriction enzyms BspEI/SalI and introduced into the plasmid pEF-DHFR+CTI + CD80-M79scFv (VL/VH) using BspEI/SalI thus replacing the M79scFv- VL/VH fragment (see Fig 25). Transfection and cell culture procedures were carried out as described above. Analysis of antigen binding was performed using the described 17-1A-ELISA (example 8.1.2.). However, no 17-1A binding activity of the alternatively arranged CD80-M79scFv-construct could be detected. Results are shown in Fig 29.

8.3. CD80 - M79 scFv (VH/VL) construct with a long $(Gly_4Ser_1)_3$ linker

First, the M79scFv (VH/VL) fragment was obtained by a two step fusion PCR as described in example 8.2. The PCR fragment encoding the VH/VL-scFv-fragment was cleaved with the restriction enzyms EcoRV/BspEI and subcloned into the Bluescript KS +CTI vector cleaved EcoRV/XmaI (see example 8.1). In a further step a longer Glyin-Serin linker $(Gly_4Ser_1)_3$ consisting of 15 aminoacids was introduced. Therefor, another oligonucleotide linker (ACCGS15BAM) which was designed to encode the $(Gly_4Ser_1)_3$ linker and to provide BspEI and BamHI compatible overhangs had to be inserted into the Bluescript KS + CTI + M79 scFv (VH/VL)(example 8.2). The nucleotide sequence of the linker is shown in Fig 30.

The plasmid Bluescript KS + CTI + M79 scFv (VH/VL) including the coding sequence of the $(Gly_4Ser_3)_3$ linker was cleaved with BspEI and SalI and the resulting DNA-fragment $(Gly_4Ser_1)_3$+M79scFv (VH/VL) was inserted into the BspEI/SalI-cleaved vector pEF-DHFR that contains the CD80-coding fragment (example8.1) thus replacing the M79scFv (VL/VH) fragment together with the short $(Gly_4Ser_1)_1$ linker(see Fig 25). For transfection and cell culture procedure example 8.1.. Antigen specific binding was analysed by 17-1A ELISA as described above

(example 8.1.1) and detection of functional recombinant protein in the cell-culture supernatant was performed with an anti His-tag antibody followed by an anti mouse IgG (Fc) antibody (compare example 8.1.1) The anti-17-1A/anti-CD3 bispecific-single-chain antibody (Mack et.al.Proc.Natl.Acad.Sci.. 92 (1995) 7021-7025) served as positve control. Development of the ELISA and measurement of the OD values was carried out as described above(example 8.1.1) However, no antigen binding was detectable. Results are shown in Fig 31.

Table 1

Antibody	Type	Company	Cat.No	Dilution
anti human CD54	mouse Ig1	Immunotech	0544	1:1000
anti human CD58	mouse IgG2	Immunotech	0861	1:1000
anti human CD80	mouse IgG1	Immunotech	1449	1:1000
anti human CD86	mouse IgG1	R&D Systems	MB141	1:1000
anti mouse IgG peroxidase conj.	goat	Dianova, Hamburg	115-037-071	1:5000
anti human Ck biotinylated	goat	Pierce	31780	1:1000
Streptavidin		Dako, Hamburg	P0347	1:1000

Table 2

T-cells	concentrations of M79scFvCK/CD80CH 1Heterominibody	concentration of the bispecific single chain antibody M79scFv-antiCD3scFv
CD4+CD45RO-	500ng/ml	250ng/ml
CD4+CD45RO-	500ng/ml	50ng/ml
CD4+CD45RO-	500ng/ml	10ng/ml
CD4+CD45RO-	500ng/ml	2ng/ml
CD4+CD45RO-	500ng/ml	0ng/ml
CD4+CD45RO-	0ng/ml	250ng/ml
CD4+CD45RO-	0ng/ml	50ng/ml
CD4+CD45RO-	0ng/ml	10ng/ml
CD4+CD45RO-	0ng/ml	2ng/ml
CD4+CD45RO-	0ng/ml	0ng/ml
CD4+CD45RO- without 17-1A positive CHO cells	500ng/ml	250ng/ml
PBMC	500ng/ml	250ng/ml
PBMC	0ng/ml	250ng/ml
PBMC	0ng/ml	0ng/ml

Table 3

cells	HeterominibodyCD80 conc.	M79SCFV-ANTICD3 conc.
CD8+CD45RO-	500ng/ml	250ng/ml
CD8+CD45RO-	500ng/ml	50ng/ml
CD8+CD45RO-	500ng/ml	10ng/ml
CD8+CD45RO-	500ng/ml	2ng/ml
CD8+CD45RO-	500ng/ml	0ng/ml
CD8+CD45RO-	0ng/ml	250ng/ml
CD8+CD45RO-	0ng/ml	50ng/ml
CD8+CD45RO-	0ng/ml	10ng/ml
CD8+CD45RO-	0ng/ml	2ng/ml
CD8+CD45RO-	0ng/ml	0ng/ml
CD8+CD45RO- without 17-1A positive CHO cells	500ng/ml	250ng/ml
PBMC	500ng/ml	250ng/ml
PBMC	0ng/ml	250ng/ml
PBMC	0ng/ml	0ng/ml

Table 4

Antibody	Type	Conjugation	Company	Dilution
anti humanCD45R0	murine IgG2a	Fluorescein-isothiocyanat (FITC)	DAKO, Hamburg, Germany	1:50
anti human CD45RA	murine IgG1	R-Phyco-erythrin (PE)	Coulter Immunotech	1:50
Isotyp IgG2a	murine IgG2a	FITC	Coulter Immunotech	1:25
Isotyp IgG1	murine IgG1	PE	Coulter Immunotech	1:100
anti human CD4	murine IgG2a	TRI-COLOR®	Caltag, distributed by Medac, Hamburg	1:50
anti human CD8	murine IgG2a	TRI-COLOR®	Caltag, distributed by Medac, Hamburg	1:200

CLAIMS

1. A multifunctional compound, produceable in a mammalian host cell as a secretable and fully functional heterodimer of two polypeptide chains, wherein one of said polypeptide chains comprises, as the only constant region domain of an immunoglobulin heavy chain the C_H1-domain and the other polypeptide chain comprises the constant C_L-domain of an immunoglobulin light chain, wherein said polypeptide chains further comprise, fused to said constant region, domains at least two (poly)peptides having different receptor or ligand functions, wherein further at least two of said different (poly)peptides do not have an intrinsic affinity for one another and wherein said polypeptide chains are linked via said constant domains.

2. The multifunctional compound of claim 1, wherein the functional domains, having receptor or ligand function, are C-and/or N-terminally linked to one or both of said constant immunoglobulin domains.

3. The multifunctional compound of claim 1 or 2, comprising at least three functional domains, having receptor or ligand function.

4. The multifunctional compound of claim 1 or 2, wherein at least two domains, having receptor or ligand function, are N-terminally linked to said constant C_H1 or C_L domains.

5. The multifunctional compound of any one of claims 1 to 4, wherein at least one of said domains, having receptor or ligand function, is in the format of a scFv-fragment or a functional part thereof.

6. The multifunctional compound of any one of claims 1 to 5, wherein at least one of said domains, having receptor- or ligand function, is a T-cell co-stimulatory ligand, an antigen binding region specific for a tumor associated antigen, or a proteinaceous compound providing the primary activation signal for T-cells.

7. The multifunctional compound of any one of claims 5 or 6, wherein said scFv fragment or said functional part thereof comprise the V_H and the V_L regions of the murine anti 17-1A antibody M79, the V_H and the V_L regions of the anti-Lewis Y antibody, as shown in Fig. 6, or the V_H and the V_L regions of the anti-CD3 antibody TR66.

8. The multifunctional compound of claim 6, wherein the T-cell co-stimulatory ligand is a cell surface molecule or a fragment thereof expressed on antigen-presenting cells (APC).

9. The multifunctional compound of claim 8, wherein the antigen-presenting cell is a dendritic cell.

10. The multifunctional compound of claim 8, wherein the cell surface molecule is selected from the group consisting of B7-1, B7-2, ICAM-1, ICAM-2, ICAM-3, LFA-3 and CD137-ligand.

11. The multifunctional compound of any one of claims 1 to 10, wherein said constant domain of an immunoglobulin light chain is of the κ type.

12. The multifunctional compound of any one of claims 1 to 11, wherein said constant immunoglobulin domains and said functional receptor-ligand domains are connected by a polypeptide linker.

13. The multifunctional compound of claim 12, wherein said polypeptide linker comprises an Ig-hinge region or a plurality of glycine, alanine and/or serine.

14. The multifunctional compound of claim 13, wherein said Ig-hinge region is an IgG hinge region.

15. The multifunctional compound of claim 14, wherein the IgG hinge region is the upper hinge region of human IgG_3.

16. The multifunctional compound of any one of claims 1 to 15, wherein said C_H1 domain is limited to a histidine tag, GST, Staphylococcus protein A, Lex A, a FLAG-tag or a MYC-tag.

17. A polynucleotide encoding one and/or two polypeptide chains of the multifunctional compound as defined in any one of claims 1 to 16.

18. A vector comprising at least one polynucleotide of claim 17.

19. A mammalian host cell comprising at least one vector of claim 18.

20. The mammalian host cell of claim 19 which is a CHO cell or a myeloma cell.

21. A method of producing the multifunctional compound of any one of claims 1 to 16 comprising culturing the host cell of claim 19 or 20 under conditions that allow the synthesis and secretion of said multifunctional compound, and recovering said multifunctional compound from the culture.

22. A pharmaceutical composition comprising the multifunctional compound of any one of claims 1 to 16, the polynucleotide of claim 17, and/or the vector of claim 18 and, optionally, a proteinaceous compound capable of providing the primary activation signal for T-cells and a pharmaceutically acceptable carrier and/or the diluent and/or excipient.

23. A diagnostic composition comprising the multifunctional compound of any one of claims 1 to 16, the polynucleotide of claim 17, and/or the vector of claim 18 and, optionally, a proteinaceous compound capable of providing the primary activation signal for T-cells and, optionally, suitable means for detection.

24. Use of the multifunctional compound of any one of claims 1 to 16, the polynucleotide of claim 17 and/or the vector of claim 18 for the preparation of a pharmaceutical composition for preventing and/or treating malignant cell growth.

25. The use of claim 24, wherein the malignant cell growth is related to malignancies of hemapoietic cells or to solid tumors.

26. The use of claim 25, wherein said malignancies of hematopoietic cells are lymphomas or leukemias.

27. The use of claim 25, wherein said solid tumors are carcinomas, melanomas or sarcomas.

28. A kit comprising the multifunctional compound of any one of claims 1 to 16 and, optionally, a proteinaceous compound capable of providing the primary activation signal for T-cells.

29. The pharmaceutical composition of claim 22, the diagnostic composition of claim 23 or the kit of claim 28, wherein the proteinaceous compound capable of providing the primary activating signal for T-cells is a bispecific antibody interacting with the T-cell antigen CD3.

Abstract

The present invention relates to a multifunctional compound, produceable in a mammalian host cell as a secretable and fully functional heterodimer of two polypeptide chains, wherein one of said polypeptide chains comprises, as the only constant region domain of an immunoglobulin heavy chain the C_H1-domain and the other polypeptide chain comprises the constant C_L-domain of an immunoglobulin light chain, wherein said polypeptide chains further comprise, fused to said constant region, domains at least two (poly)peptides having different receptor or ligand functions, wherein further at least two of said different (poly)peptides do not have an intrinsic affinity for one another and wherein said polypeptide chains are linked via said constant domains. Preferably, said domains, having receptor or ligand function, are in the format of a scFv-fragment. Most preferably, said scFV-fragment comprise the V_H and the V_L regions of the murine anti 17-1A antibody M79, the V_H and the V_L regions of the anti-Lewis Y antibody, as shown in Fig. 6, or the V_H and the V_L regions of the anti-CD3 antibody TR66. Furthermore, the present invention relates to polynucleotides encoding said polypeptide chains as well as vectors comprising said polynucleotides and host cells transformed therewith as well as the use of the above embodiments for the production of said multifunctional compounds. In addition, pharmaceutical and diagnostic compositions are provided. comprising any of the afore-described multifunctional compounds, polynucleotides or vectors. Described is also the use of the afore-mentioned multifunctional compound for preventing and/or treating malignant cell growth, related to malignancies of hemapoietic cells or to solid tumors.

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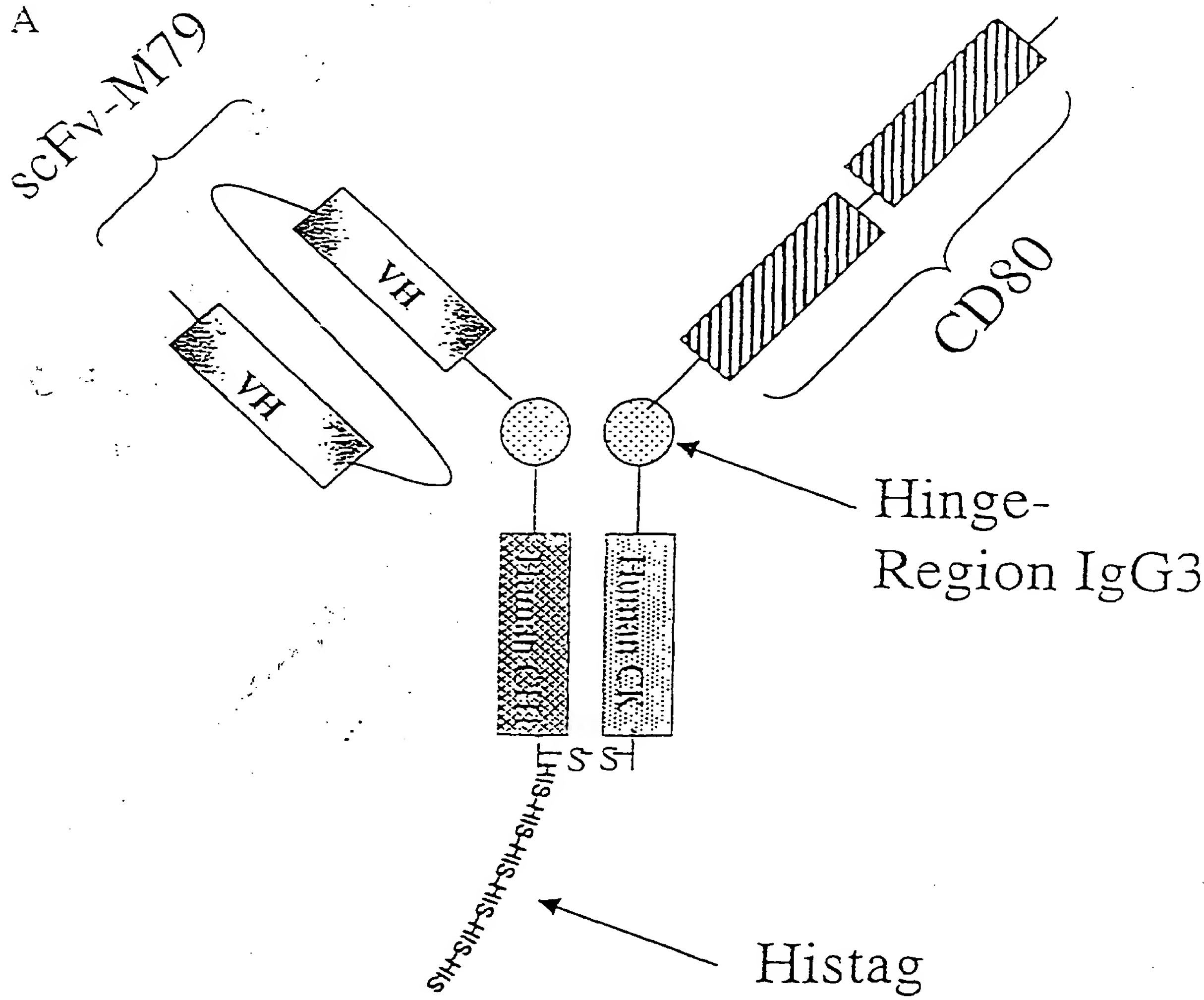
Figure 1

A



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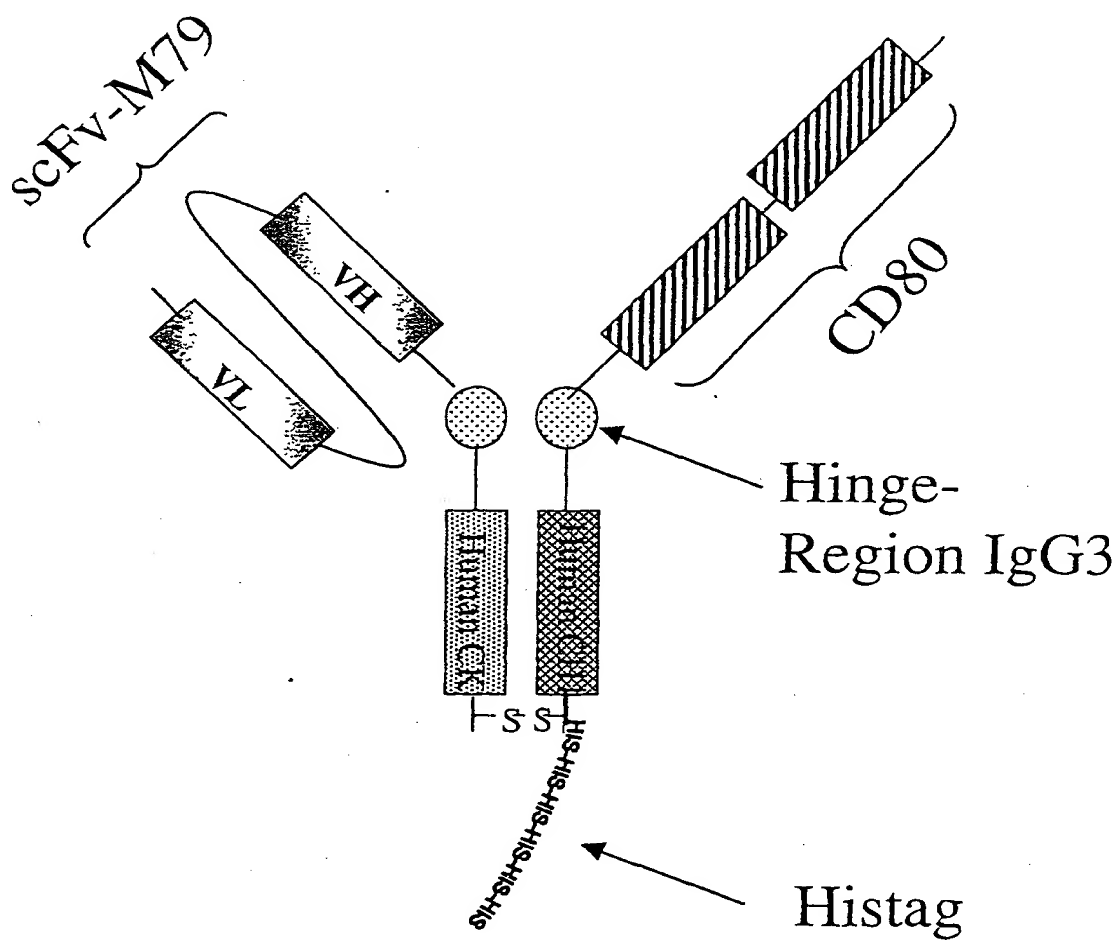
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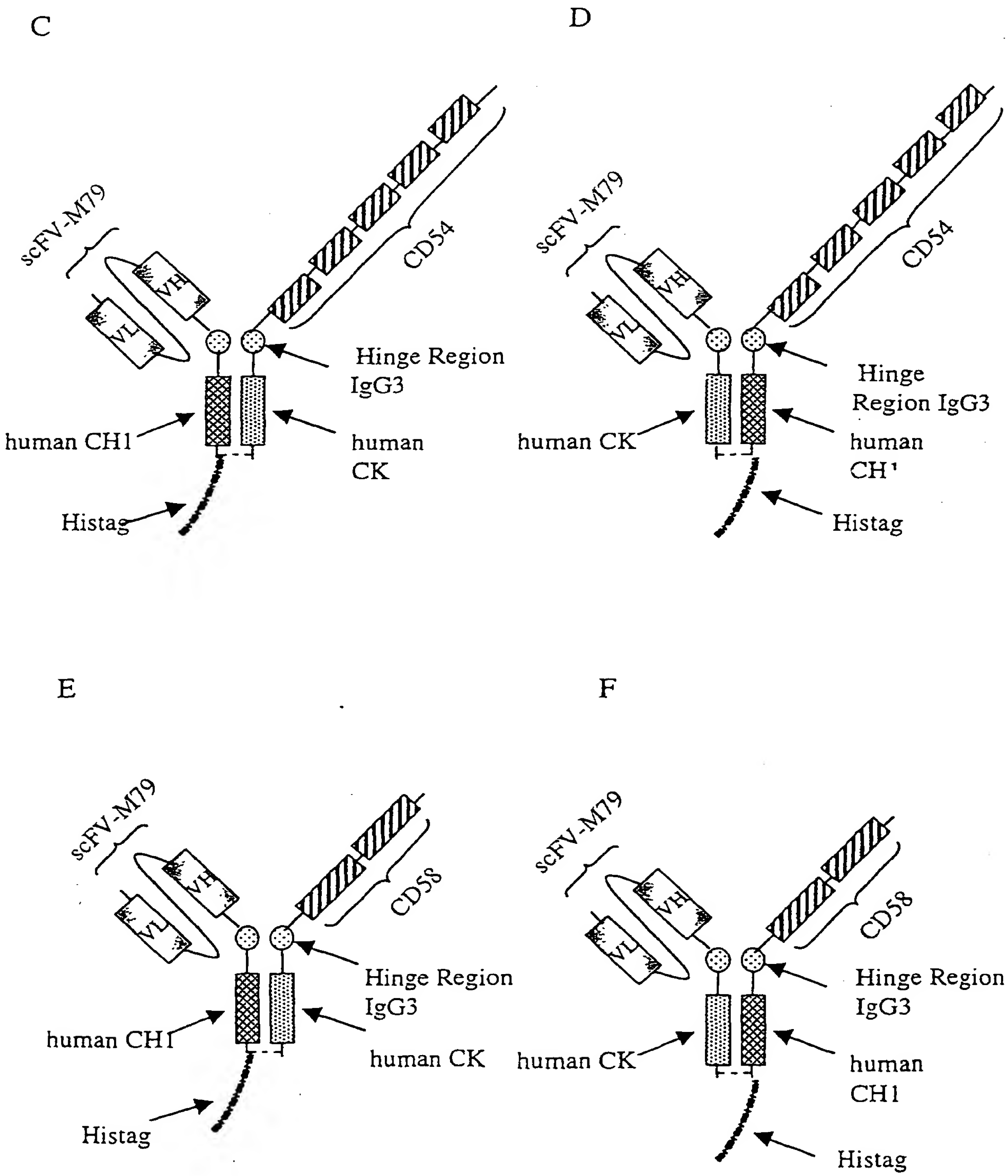
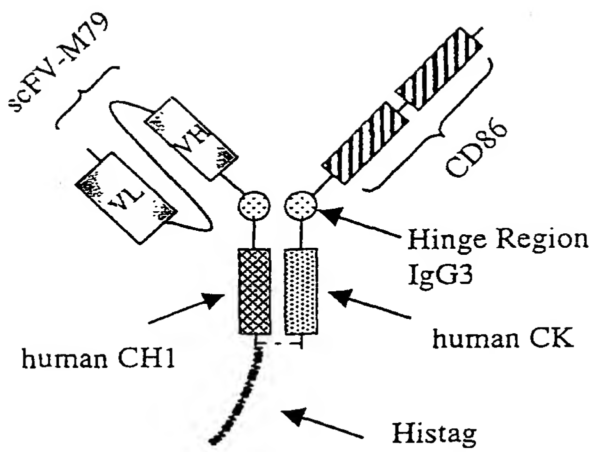
VH
VL
CD80
Hinge-
Region IgG3
Human Cκ
Human CH1
S S
His-His-His-His-His-His
Histag


C
scFV-M79
VH
VL
CD54
Hinge Region IgG3
human CH1
human CK
Histag
D
scFV-M79
VH
VL
CD54
Hinge Region IgG3
human CK
human CH1
Histag
E
scFV-M79
VH
VL
CD58
Hinge Region IgG3
human CH1
human CK
Histag
F
scFV-M79
VH
VL
CD58
Hinge Region IgG3
human CK
human CH1
Histag

G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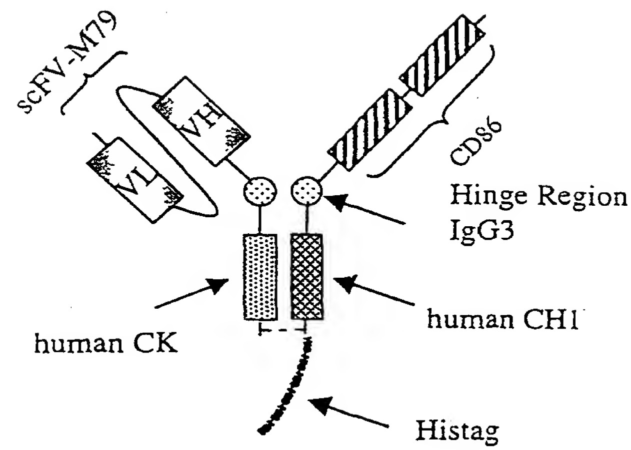
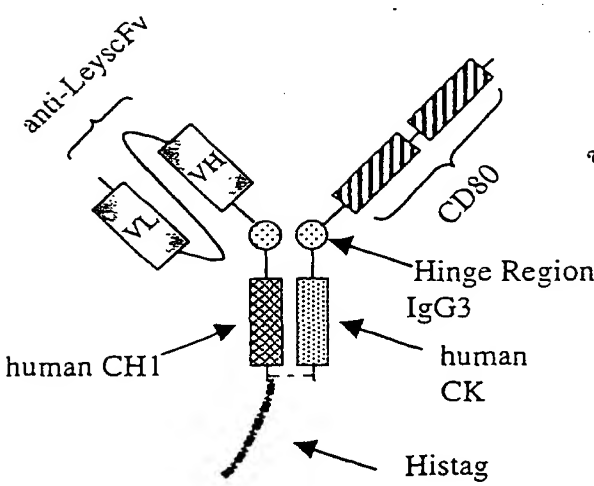
VH
VL
CD86
Hinge Region
IgG3
human CH1
human CK
Histag

H


scFV-M79
VH
VL
CD86
Hinge Region
IgG3
human CK
human CH1
Histag

I


anti-LeyscFv
VH
VL
CD80
Hinge Region
IgG3
human CH1
human
CK
Histag

J

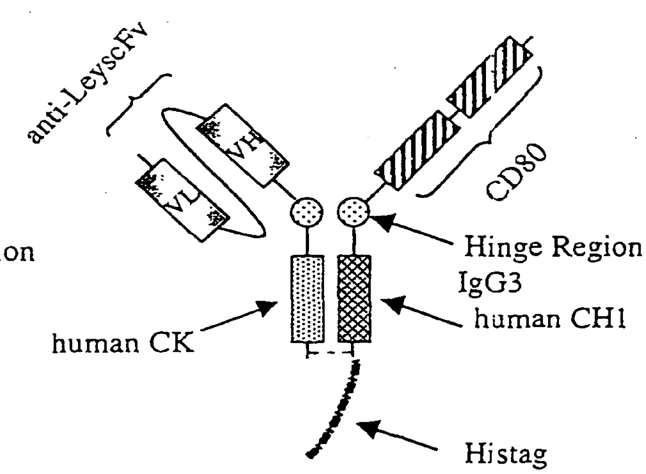

anti-LeyscFv
VH
VL
CD80
Hinge Region
IgG3
human CK
human CH1
Histag

Figure 2

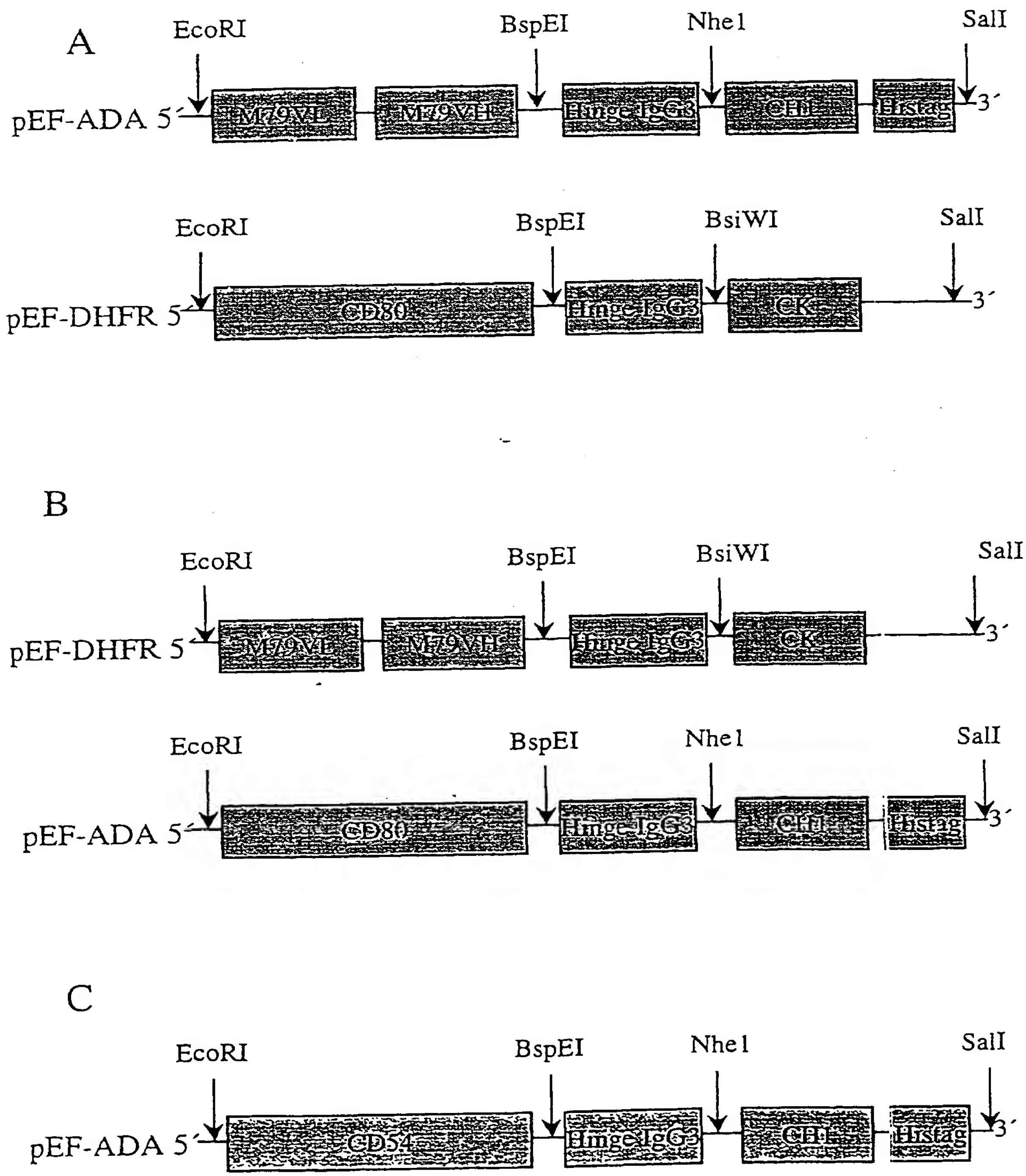
Xba I, Eco RI, Asu II, Bsp EI, BamH I, Eco RV, Xma I

5` CTA GAA TTC TTC GAA TCC GGA GGT GGT GGA TCC GAT ATC CCC

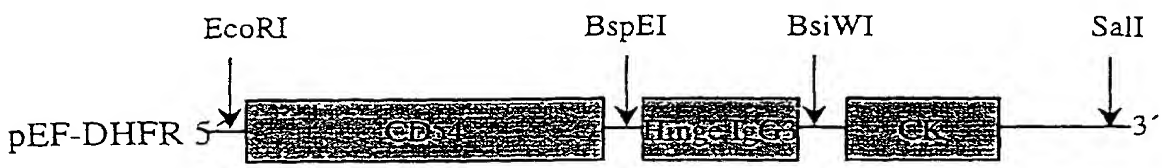
Sal I

GGG CAT CAT CAC CAT CAT CAT TGA G 3`

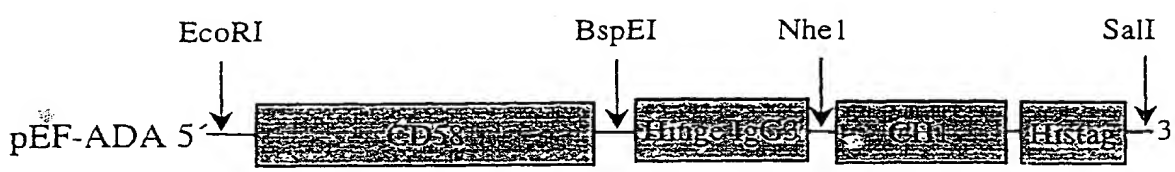
Figure 3


A
EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
M79VL
M79VH
Hinge IgG3
CH1
Histag
3´
EcoRI
BspEI
BsiWI
SalI
pEF-DHFR 5´
CD80
Hinge IgG3
CK
3´
B
EcoRI
BspEI
BsiWI
SalI
pEF-DHFR 5´
M79VL
M79VH
Hinge IgG3
CK
3´
EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD80
Hinge IgG3
CH1
Histag
3´
C
EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD54
Hinge IgG3
CH1
Histag
3´

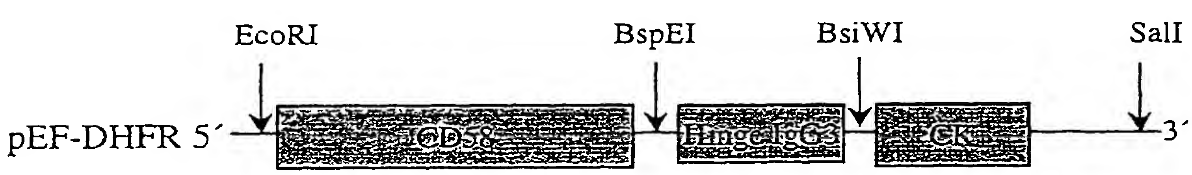
D


EcoRI
BspEI
BsiWI
SalI
pEF-DHFR 5´
CD54
Hinge IgG3
Ck
3´

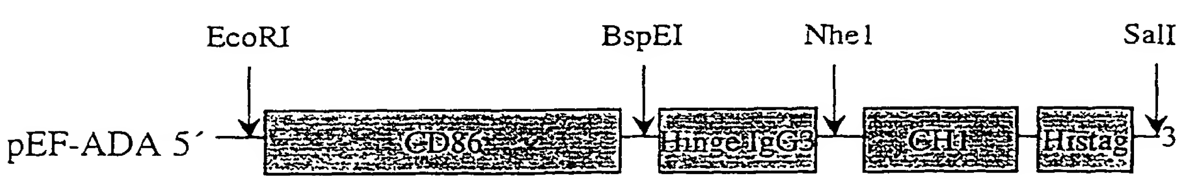
E


EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD58
Hinge IgG3
CH1
Histag
3´

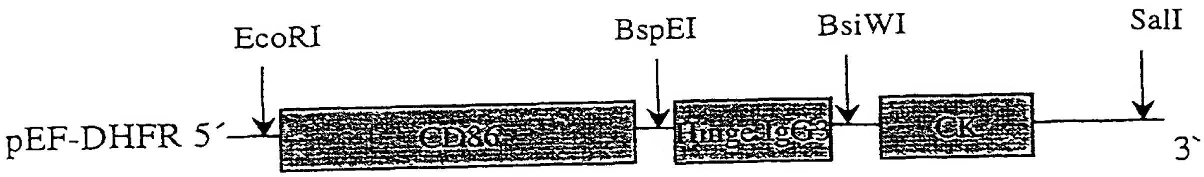
F


EcoRI
BspEI
BsiWI
SalI
pEF-DHFR 5´
CD58
Hinge IgG3
Ck
3´

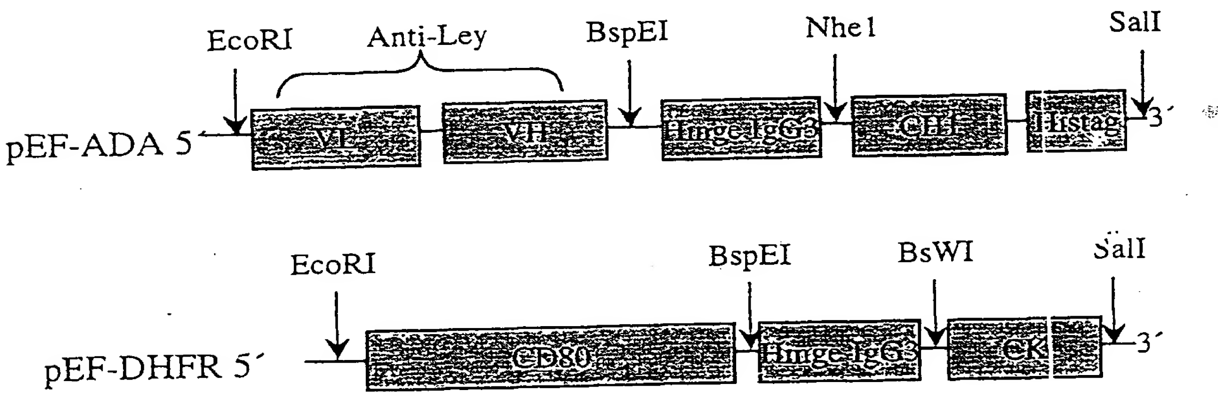
G


EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD86
Hinge IgG3
CH1
Histag
3´

H


EcoRI
BspEI
BsiWI
SalI
pEF-DHFR 5´
CD86
Hinge IgG3
CK
3`

I


EcoRI
Anti-Ley
BspEI
Nhe1
SalI
pEF-ADA 5´
VL
VH
Hinge IgG3
CH1
Histag
3´
EcoRI
BspEI
BsWI
SalI
pEF-DHFR 5´
CD80
Hinge IgG3
CK
3´

J

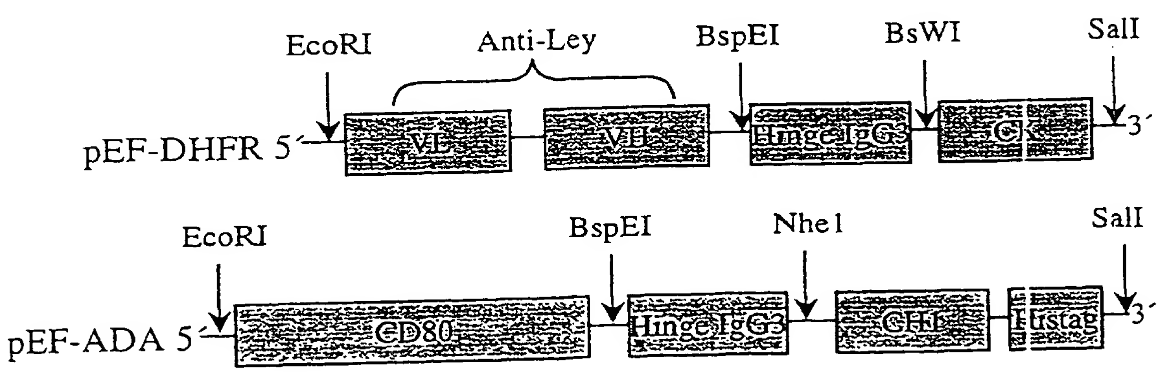
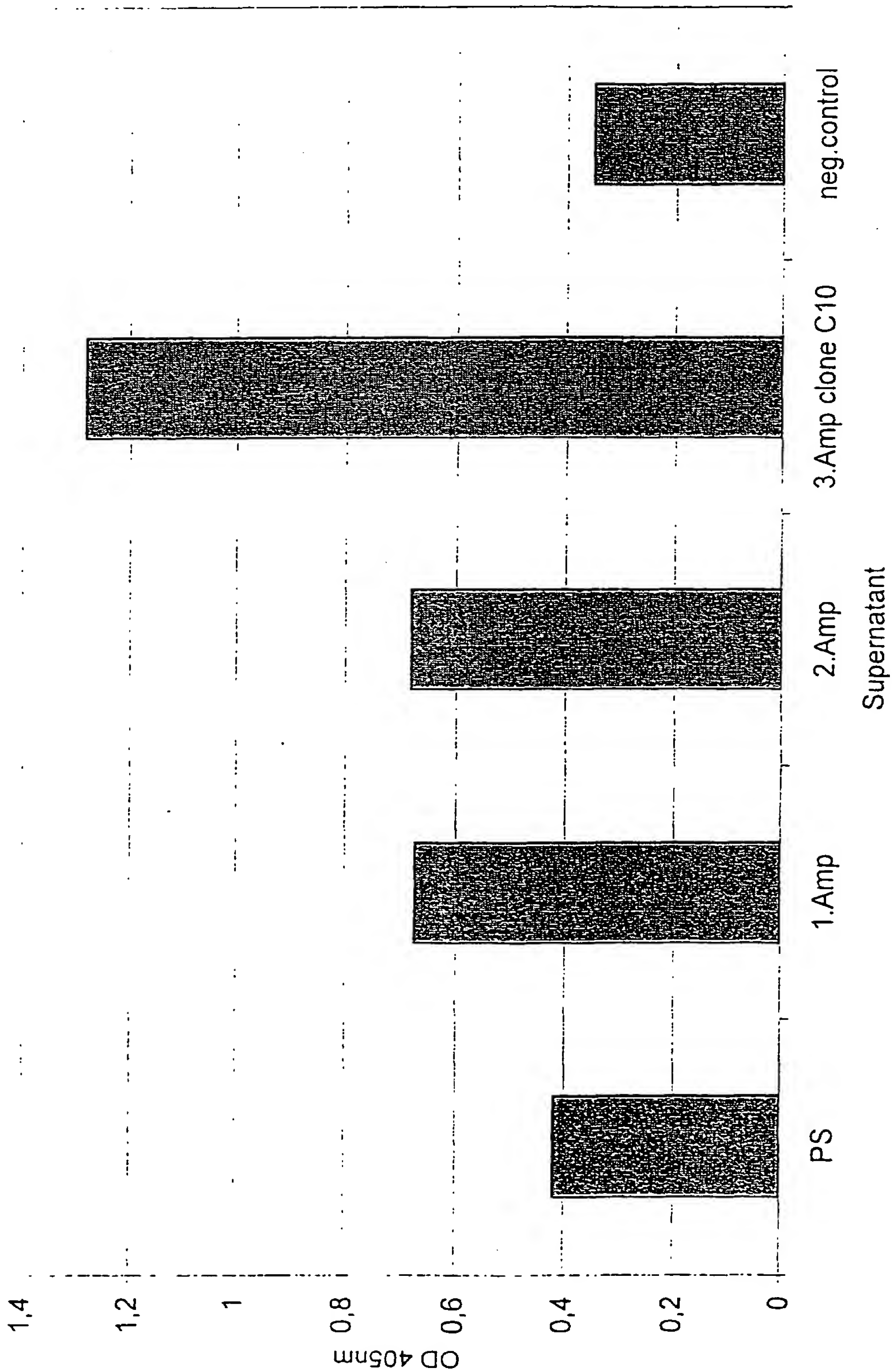

EcoRI
Anti-Ley
BspEI
BsWI
SalI
pEF-DHFR 5´
VL
VH
Hinge IgG3
Ck
3´
EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD80
Hinge IgG3
CH1
Histag
3´

Figure 4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1,2
1
0,8
0,6
0,4
0,2
0
PS
1.Amp
2.Amp
3.Amp clone C10
neg.control
Supernatant

Figure 5

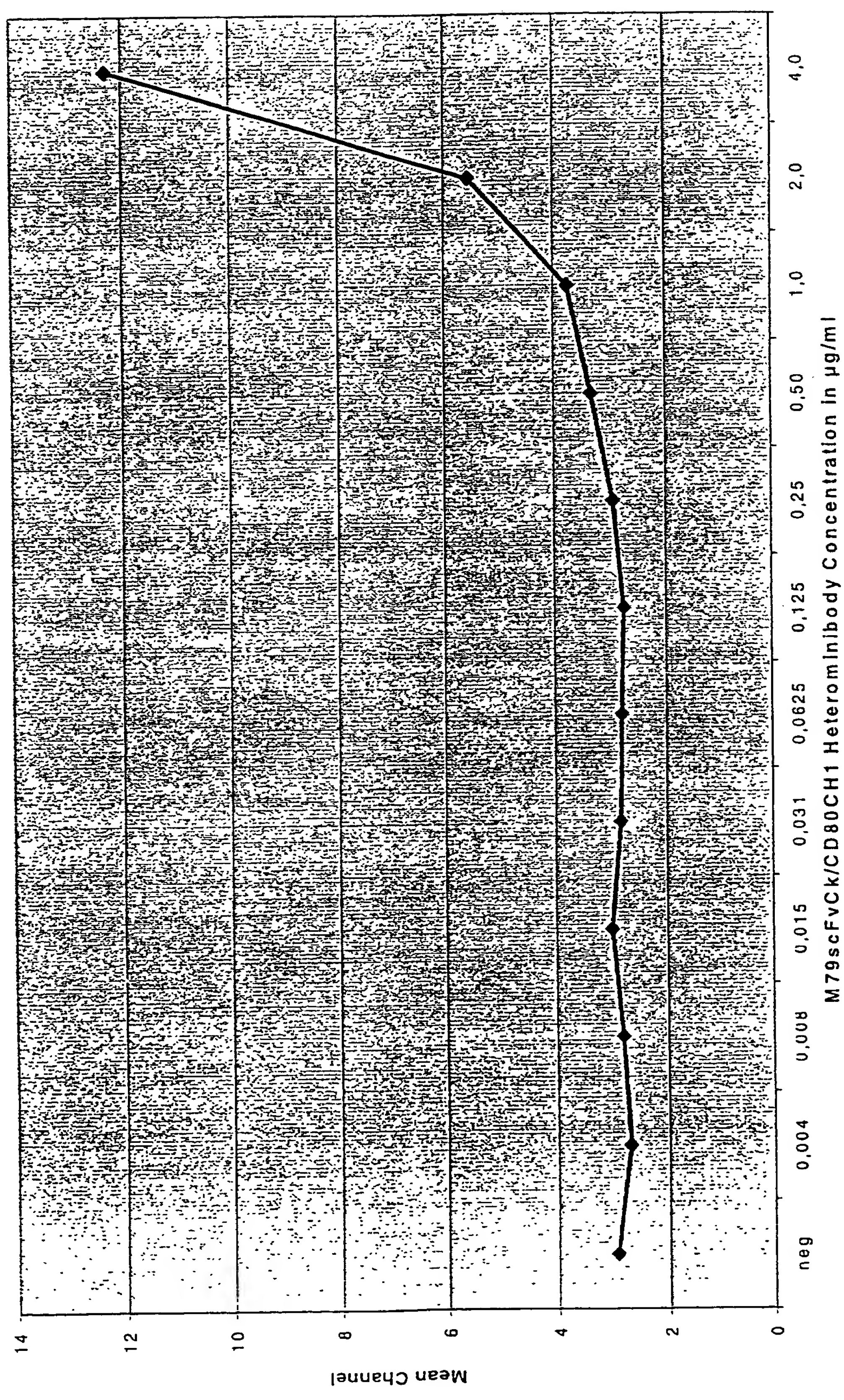

Mean Channel
0
2
4
6
8
10
12
14
neg
0,004
0,008
0,015
0,031
0,0625
0,125
0,25
0,50
1,0
2,0
4,0
M79scFvCk/CD80CH1 Heterominibody Concentration in µg/ml

Figure 6

```
    EcoRI  10           19           28           37           46           55
5' GAAT TCC ACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA
                --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                 M   G   W   S   C   I   I   L   F   L   V   A   T   A   T

           64           73           82           91          100          109
   GGT GTA CAC TCC GAT ATC GTT GTG ACT CAG GAA TCT GCA CTC ACC ACA TCA CCT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    G   V   H   S   D   I   V   V   T   Q   E   S   A   L   T   T   S   P

          118          127          136          145          154          163
   GGT GAA ACA GTC ACA CTC ACT TGT CGC TCA AGT ACT GGG GCT GTT ACA ACT AGT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    G   E   T   V   T   L   T   C   R   S   S   T   G   A   V   T   T   S

          172          181          190          199          208          217
   AAC TAT GCC AAC TGG GTC CAA GAA AAA CCA GAT CAT TTA TTC ACT GGT CTA ATA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    N   Y   A   N   W   V   Q   E   K   P   D   H   L   F   T   G   L   I

          226          235          244          253          262          271
   GGT GGT ACC AAC AAC CGA GTT CCA GGT GTT CCT GCC AGA TTC TCA GGC TCC CTG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    G   G   T   N   N   R   V   P   G   V   P   A   R   F   S   G   S   L

          280          289          298          307          316          325
   ATT GGA GAC AAG GCT GCC CTC ACC ATC ACA GGG GCA CAG ACT GAG GAT GAG GCA
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    I   G   D   K   A   A   L   T   I   T   G   A   Q   T   E   D   E   A

          334          343          352          361          370          379
   ATA TAT TTC TGT GCT CTA TGG TAC AGC AAC CAT TGG GTG TTC GGT GGA GGA ACC
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    I   Y   F   C   A   L   W   Y   S   N   H   W   V   F   G   G   G   T

          388          397          406          415          424          433
   AAA CTC GAG GTC CTA GGT GGT GGT GGT TCT GGC GGC GGC GGC TCC GGT GGT GGT
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    K   L   E   V   L   G   G   G   G   S   G   G   G   G   S   G   G   G

          442          451          460          469          478          487
   GGT TCT CAG GTC CAG CTG CAG GAG TCT GGA CCT GGC CTG GTG GCG CCC TCA CAG
   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    G   S   Q   V   Q   L   Q   E   S   G   P   G   L   V   A   P   S   Q
```

```
        496         505         514         523         532         541
AGC CTG TCC ATC ACA TGC ACC ATC TCA GGG TTC TCA TTA ACT AAG TAT GGT GTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   L   S   I   T   C   T   I   S   G   F   S   L   T   K   Y   G   V

        550         559         568         577         586         595
CAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG GTG GTG ATA TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   W   V   R   Q   P   P   G   K   G   L   E   W   L   V   V   I   W

        604         613         622         631         640         649
ACT GAT GGA GGC ACA TCC TAT AAT TCA GCT CTC AAA TCC AGA CTG AGC ATC AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   D   G   G   T   S   Y   N   S   A   L   K   S   R   L   S   I   S

        658         667         676         685         694         703
AAG GAC AAC TCC AAG AGC CAA GTT TTC TTA AAA ATG AAC AGT CTC CAA ACT GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   D   N   S   K   S   Q   V   F   L   K   M   N   S   L   Q   T   D

        712         721         730         739         748         757
GAC ACA GCC ATG TAC TAC TGT GCC AGA CAG GAT AGA TAC GAC GGT GGA ATT GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   T   A   M   Y   Y   C   A   R   Q   D   R   Y   D   G   G   I   A

                                                BspEI
        766         775         784            -------
TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCC GGA 3'
--- --- --- --- --- --- --- --- --- --- --- ---
 Y   W   G   Q   G   T   T   V   T   V   S   S
```

Figure 7

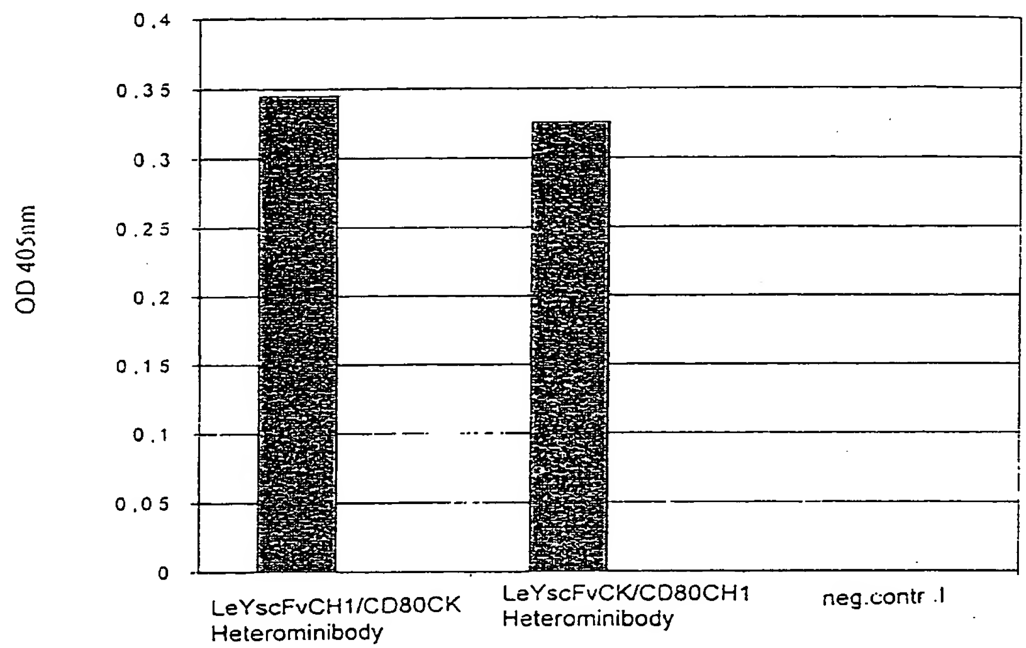
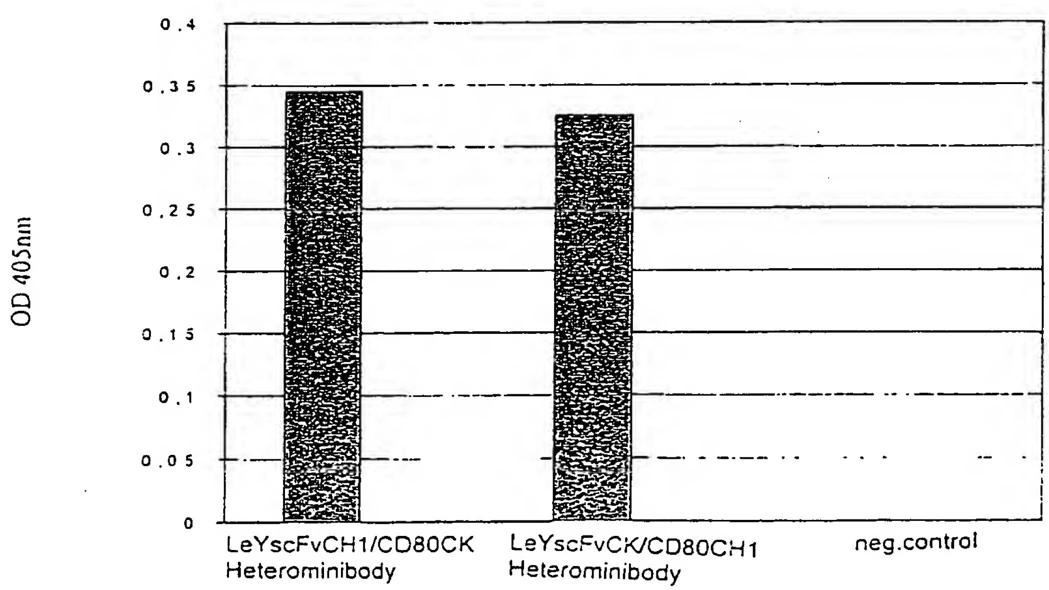
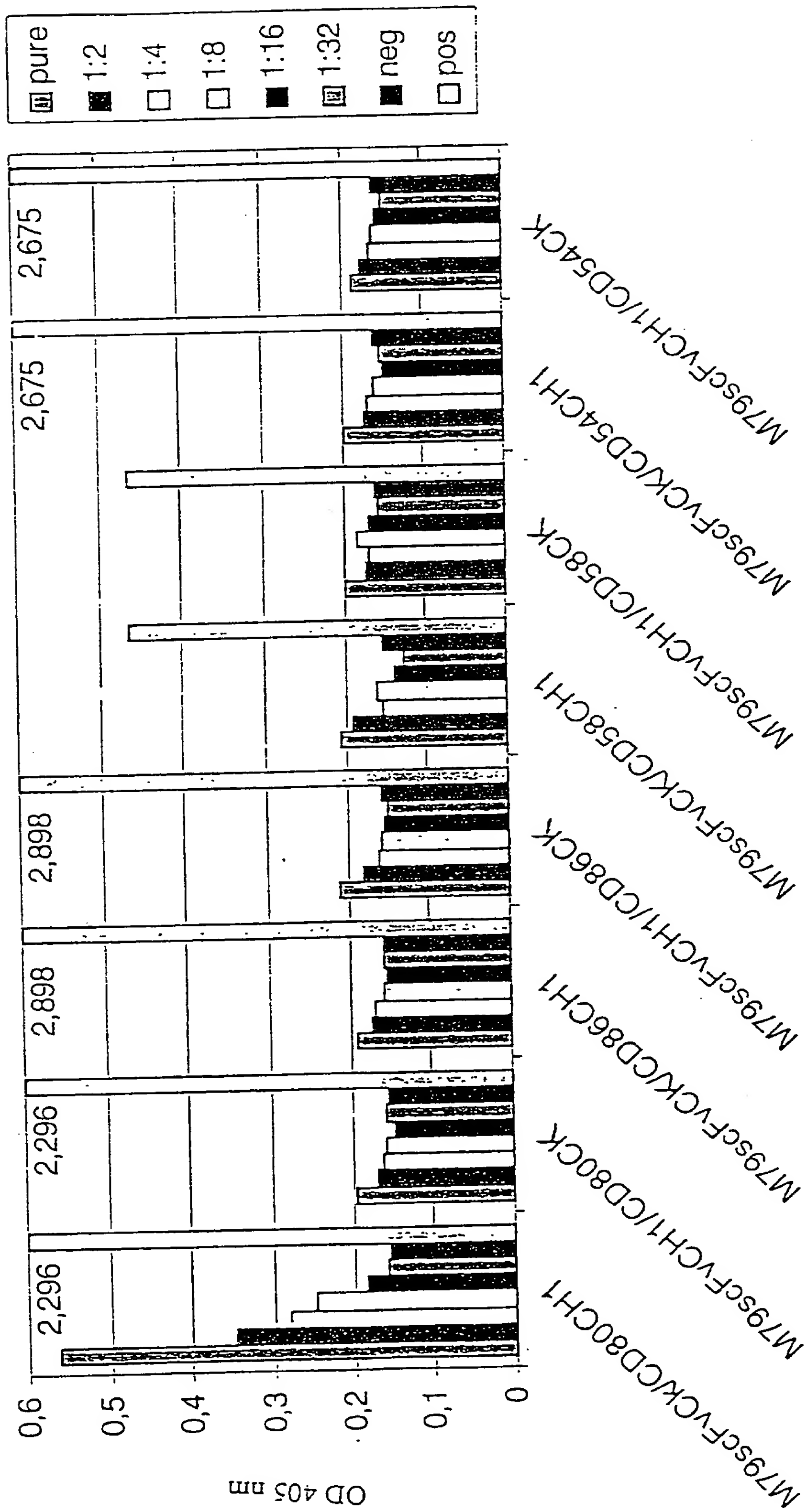


Figure 8

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1:2
1:4
1:8
1:16
1:32
neg
pos
OD 405 nm
0,6
0,5
0,4
0,3
0,2
0,1
0
2,296
2,296
2,898
2,898
2,675
2,675
M79scFvCK/CD80CH1
M79scFvCH1/CD80CK
M79scFvCK/CD86CH1
M79scFvCH1/CD86CK
M79scFvCK/CD58CH1
M79scFvCH1/CD58CK
M79scFvCK/CD54CH1
M79scFvCH1/CD54CK

Figure 9

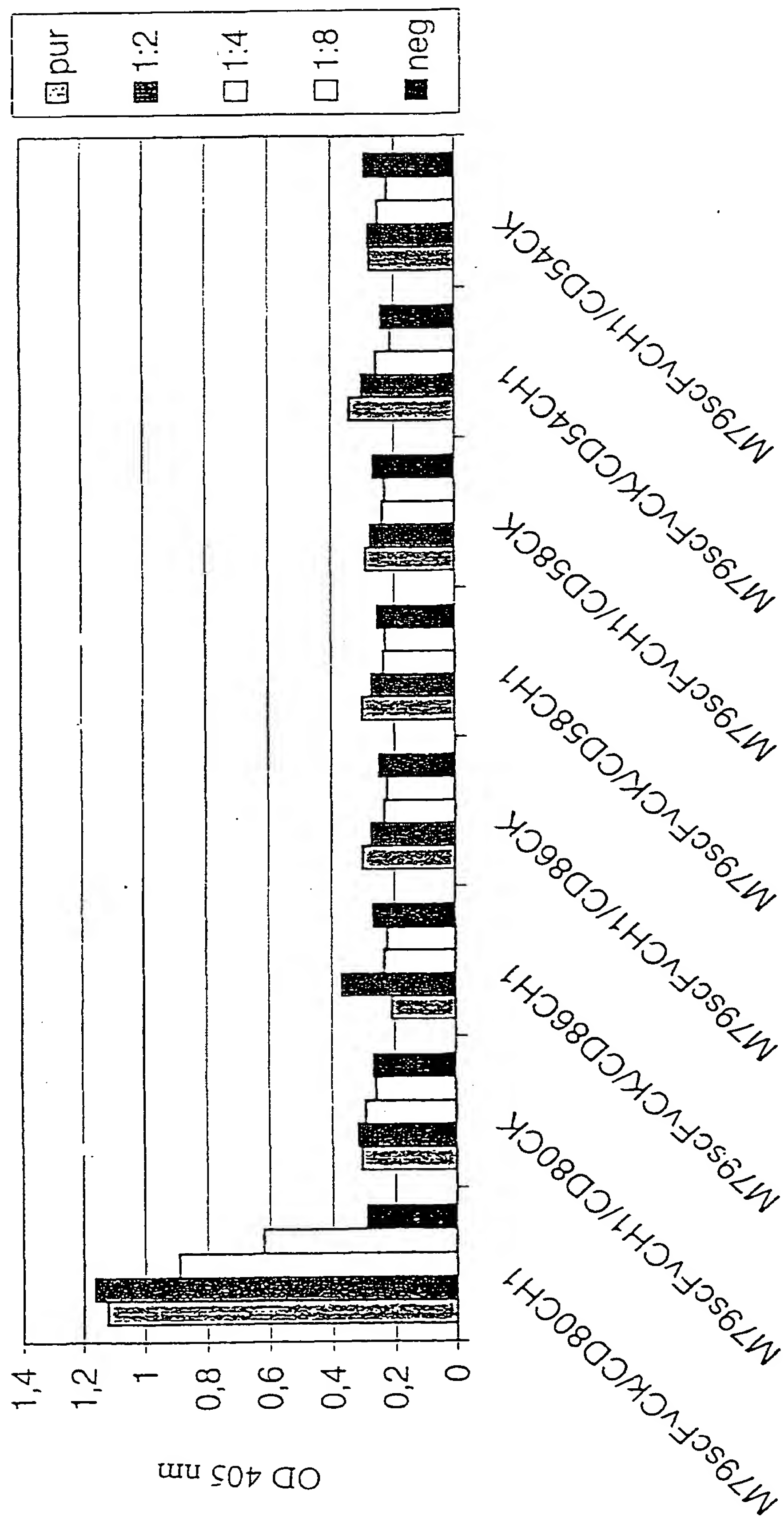
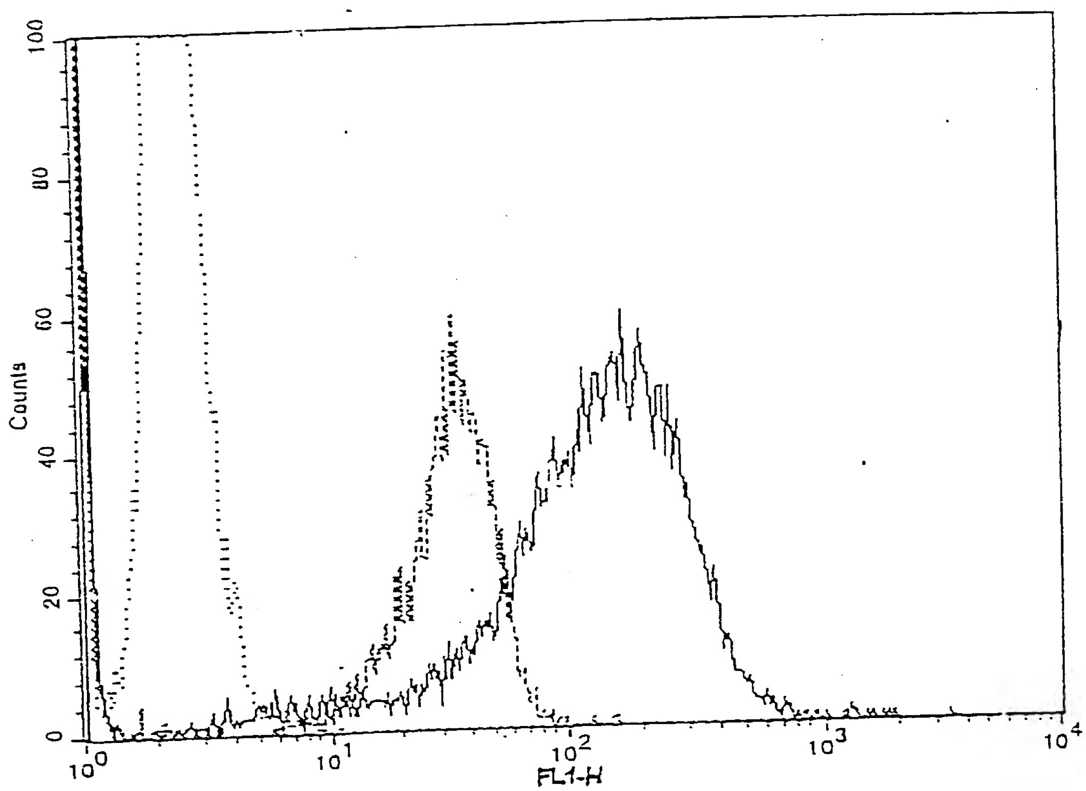

pur
1:2
1:4
1:8
neg
OD 405 nm
1,4
1,2
1
0,8
0,6
0,4
0,2
0
M79scFvCK/CD80CH1
M79scFvCH1/CD80CK
M79scFvCK/CD86CH1
M79scFvCH1/CD86CK
M79scFvCK/CD58CH1
M79scFvCH1/CD58CK
M79scFvCK/CD54CH1
M79scFvCH1/CD54CK

Figure 10

Figure 11



——— M79 on 17-1A transfected CHO cells
.... M79 on untransfected CHO cells
------ M79 on KATO cells

Figure 12

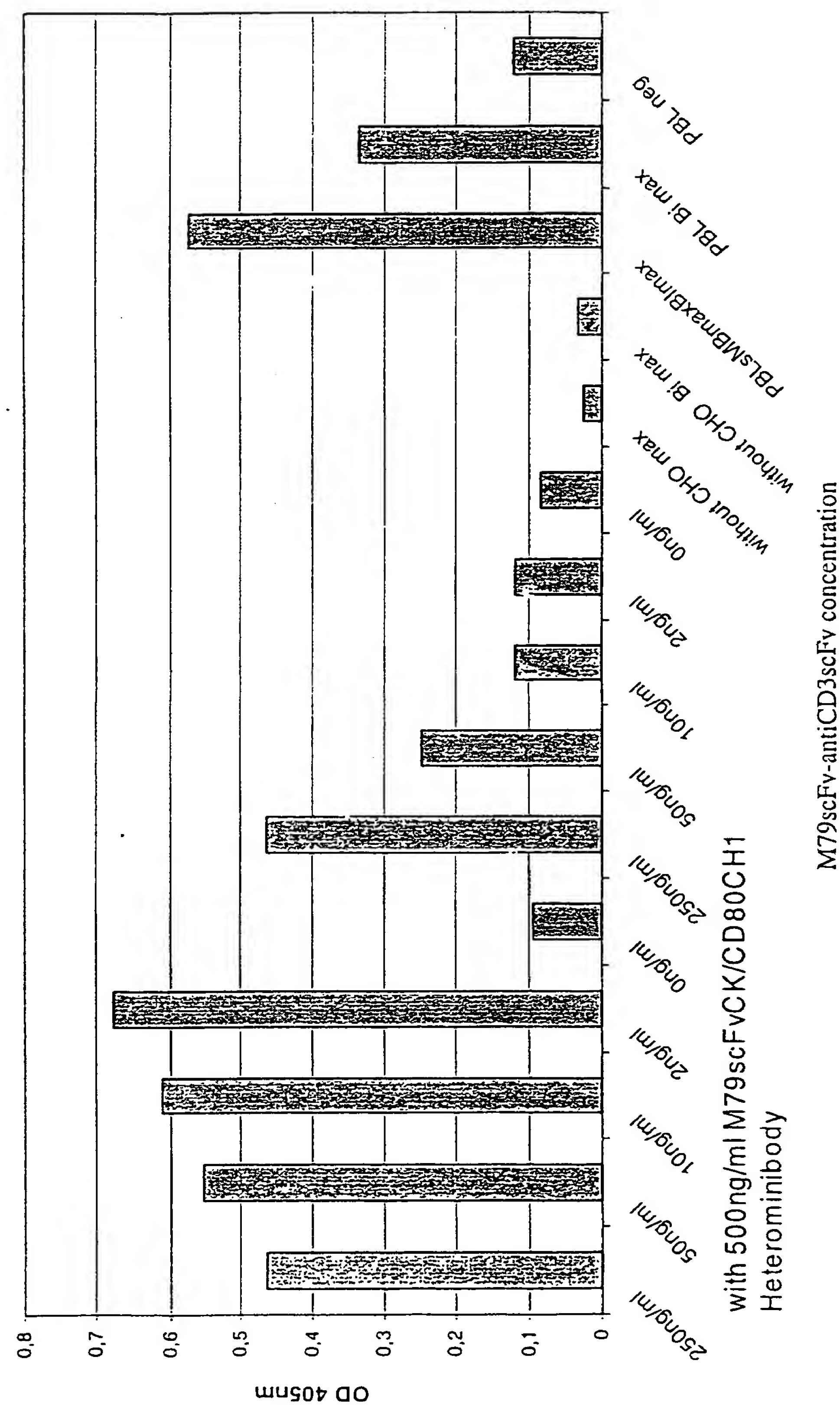

OD 405nm
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
without CHO max
without CHO Bi max
PBLsMBmaxBImax
PBL Bi max
PBL neg
with 500ng/ml M79scFvCK/CD80CH1
Heterominibody
M79scFv-antiCD3scFv concentration

Figure 13

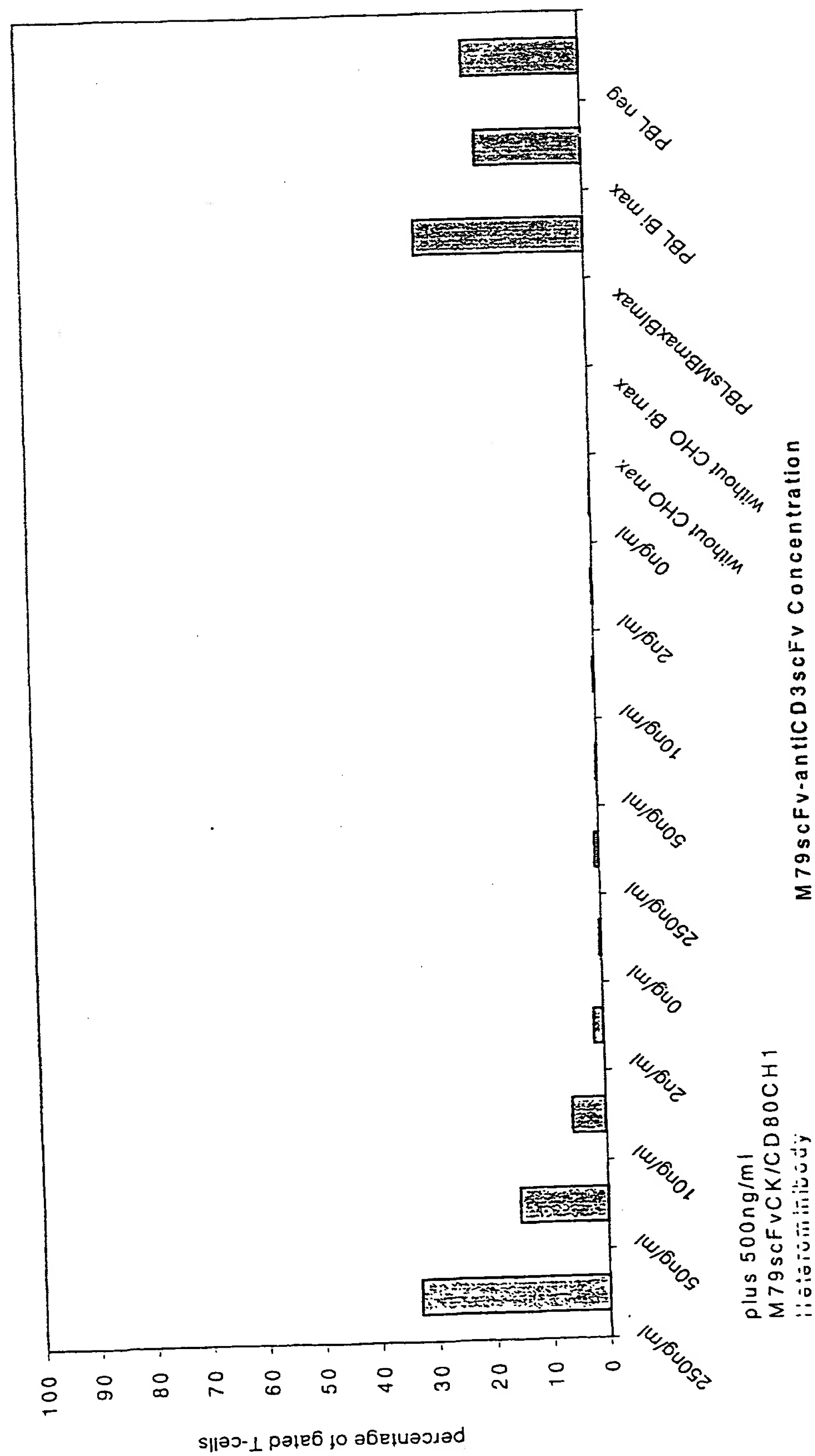
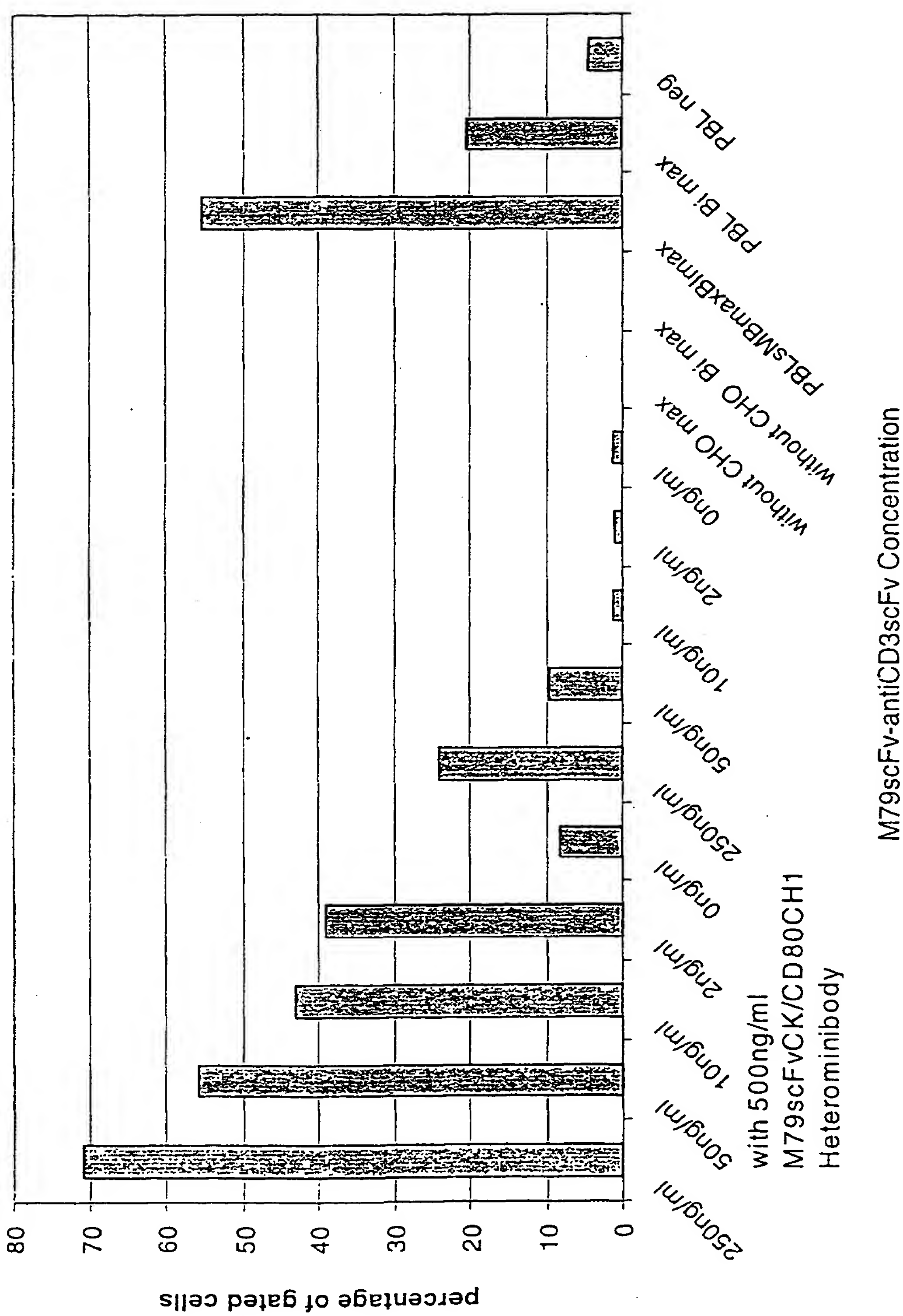
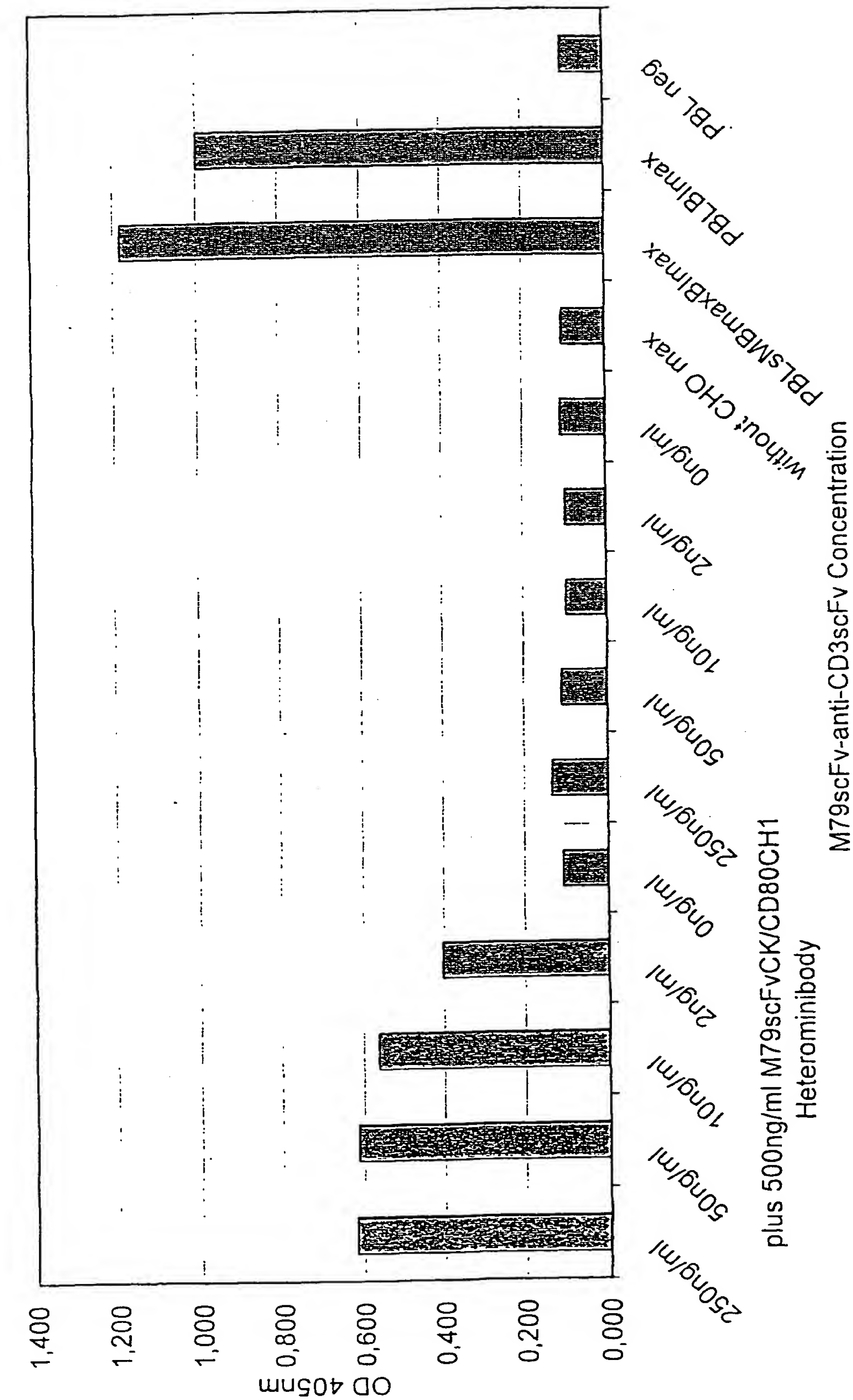

percentage of gated T-cells
0
10
20
30
40
50
60
70
80
90
100
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
without CHO max
without CHO Bi max
PBLsMBmaxBImax
PBL Bi max
PBL neg
plus 500ng/ml
M79scFvCK/CD80CH1
Heterominibody
M79scFv-antiCD3scFv Concentration

Figure14


percentage of gated cells
0
10
20
30
40
50
60
70
80
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
without CHO max
without CHO Bi max
PBLsMBmaxBImax
PBL Bi max
PBL neg
with 500ng/ml
M79scFvCK/CD80CH1
Heterominibody
M79scFv-antiCD3scFv Concentration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1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
PBL neg
PBLBlmax
PBLsMBmaxBlmax
without CHO max
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
M79scFv-anti-CD3scFv Concentration
plus 500ng/ml M79scFvCK/CD80CH1
Heterominibody

Figure 15

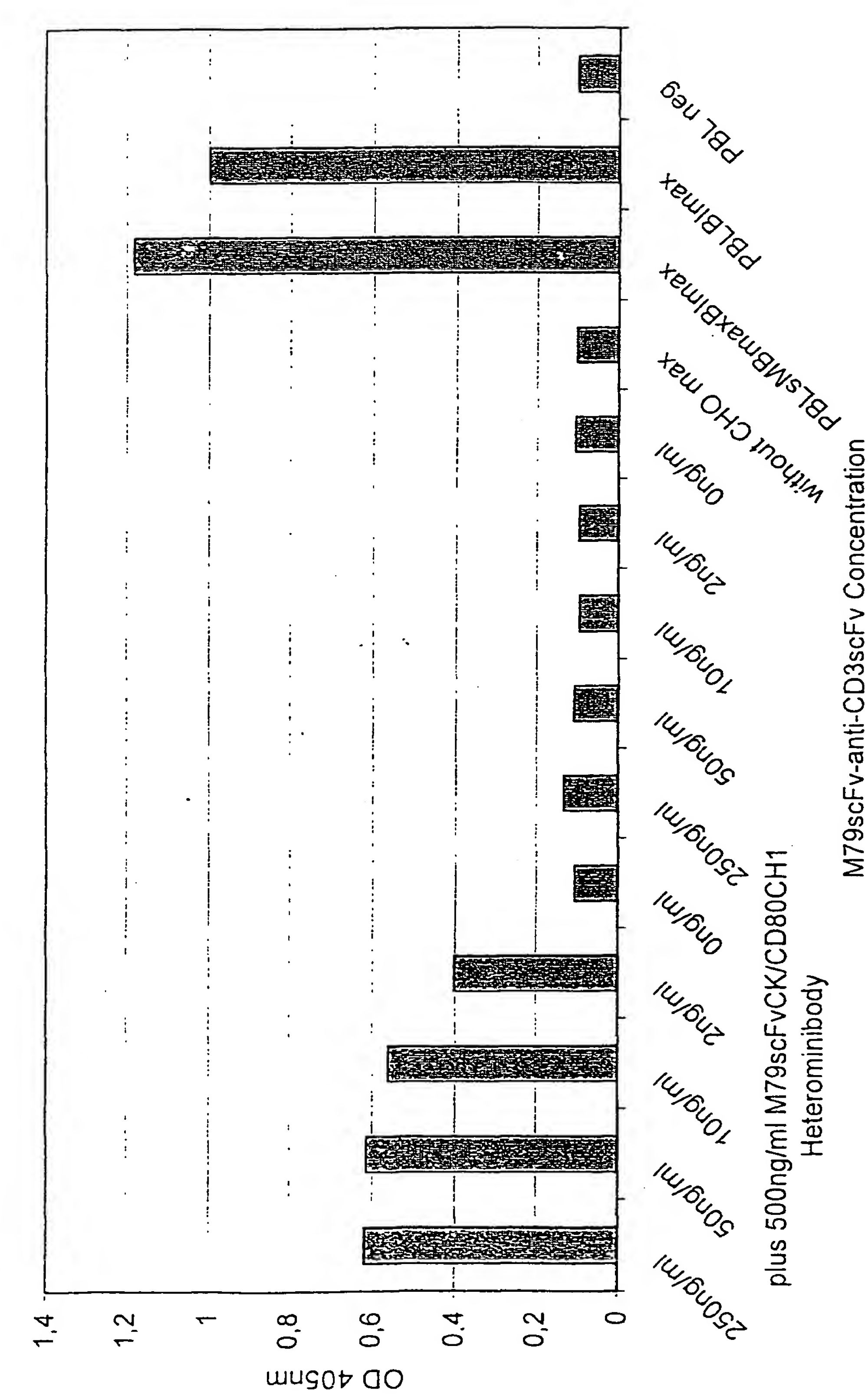
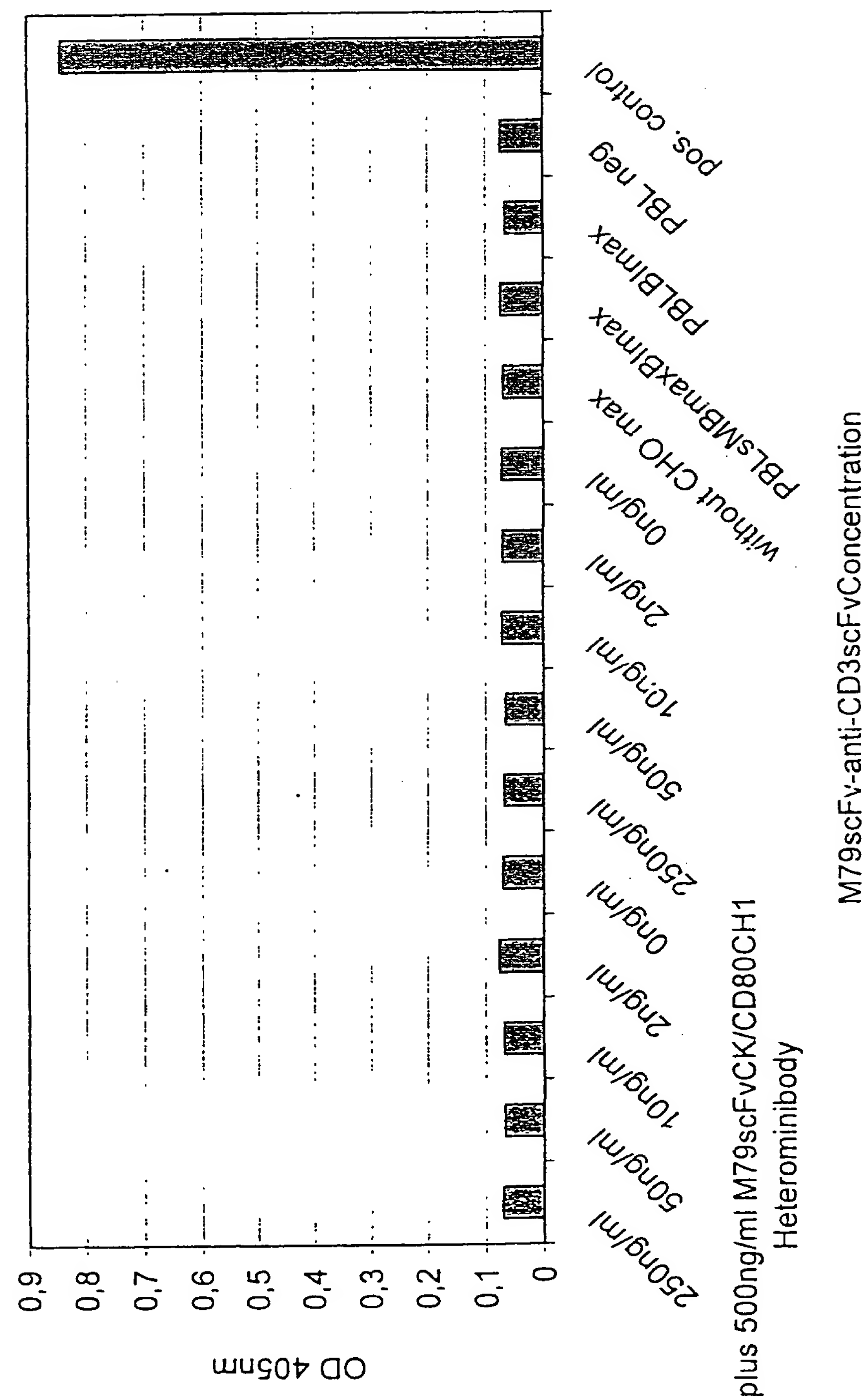

OD 405nm
1,4
1,2
1
0,8
0,6
0,4
0,2
0
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
without CHO max
PBLsMBmaxBlmax
PBLBlmax
PBL neg
plus 500ng/ml M79scFvCK/CD80CH1
Heterominibody
M79scFv-anti-CD3scFv Concentration

Figure 16

Figure17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0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
pos. control
PBL neg
PBLBlmax
PBLsMBmaxBlmax
without CHO max
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
M79scFv-anti-CD3scFvConcentration
plus 500ng/ml M79scFvCK/CD80CH1
Heterominibody

Figure18

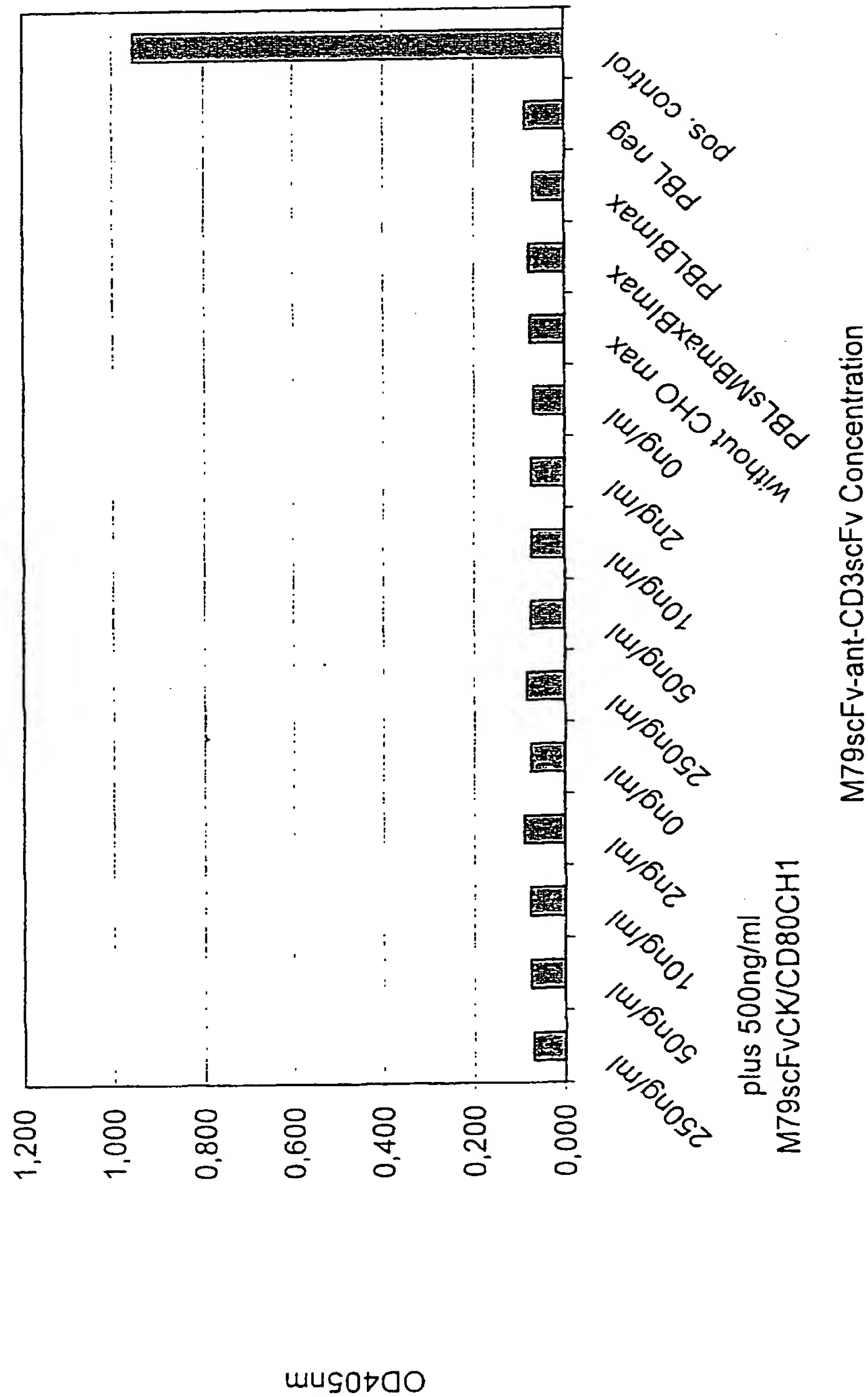
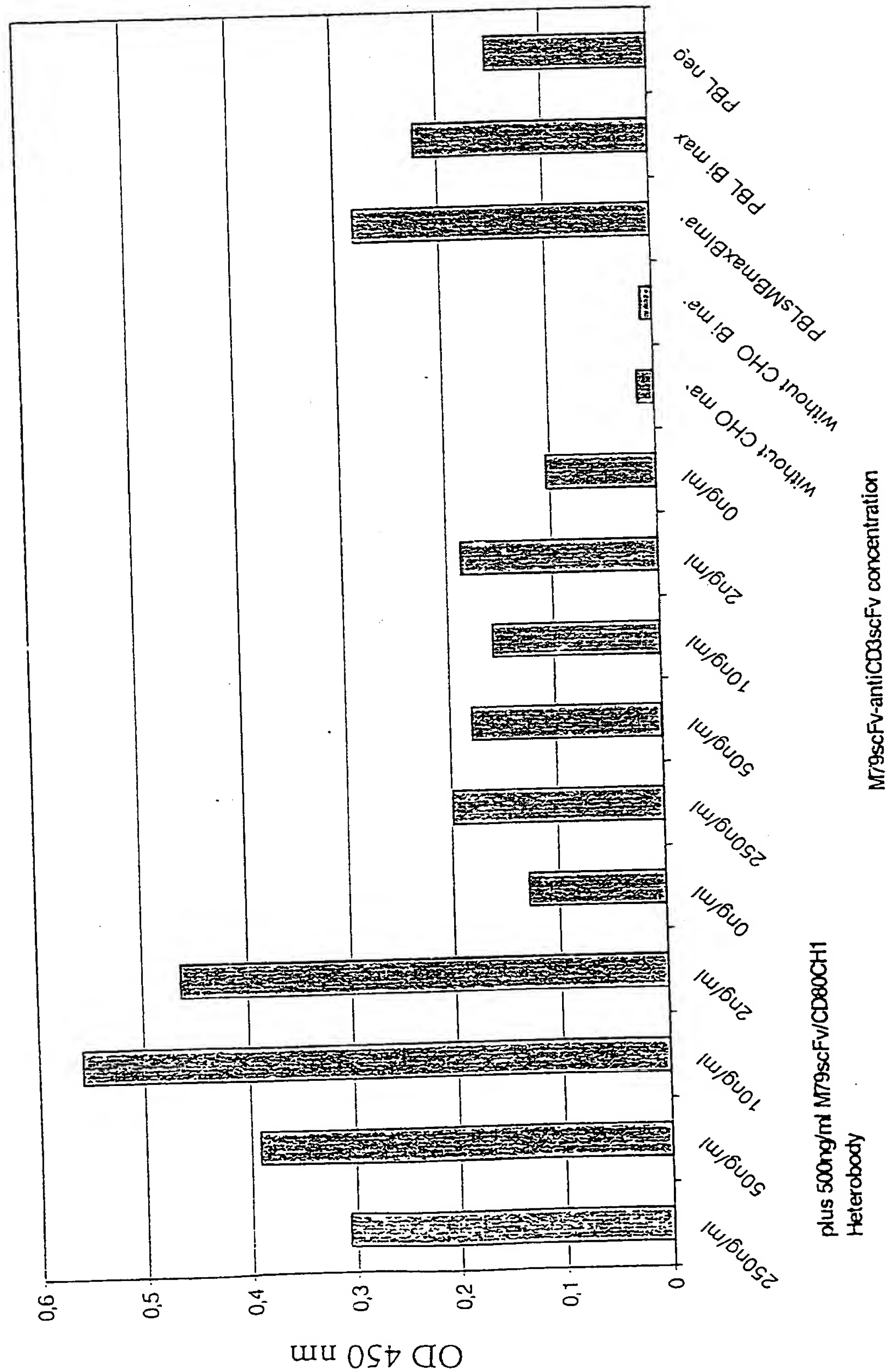

1,200
1,000
0,800
0,600
0,400
0,200
0,000
pos. control
PBL neg
PBLBlmax
PBLsMBmaxBlmax
without CHO max
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
plus 500ng/ml
M79scFvCK/CD80CH1
M79scFv-ant-CD3scFv Concentration
OD405nm

Figure 19


OD 450 nm
0
0,1
0,2
0,3
0,4
0,5
0,6
PBL neg
PBL Bi max
PBLsMBmaxBima.
without CHO Bi ma.
without CHO ma.
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
M79scFv-antiCD3scFv concentration
plus 500ng/ml M79scFv/CD80CH1
Heterobody

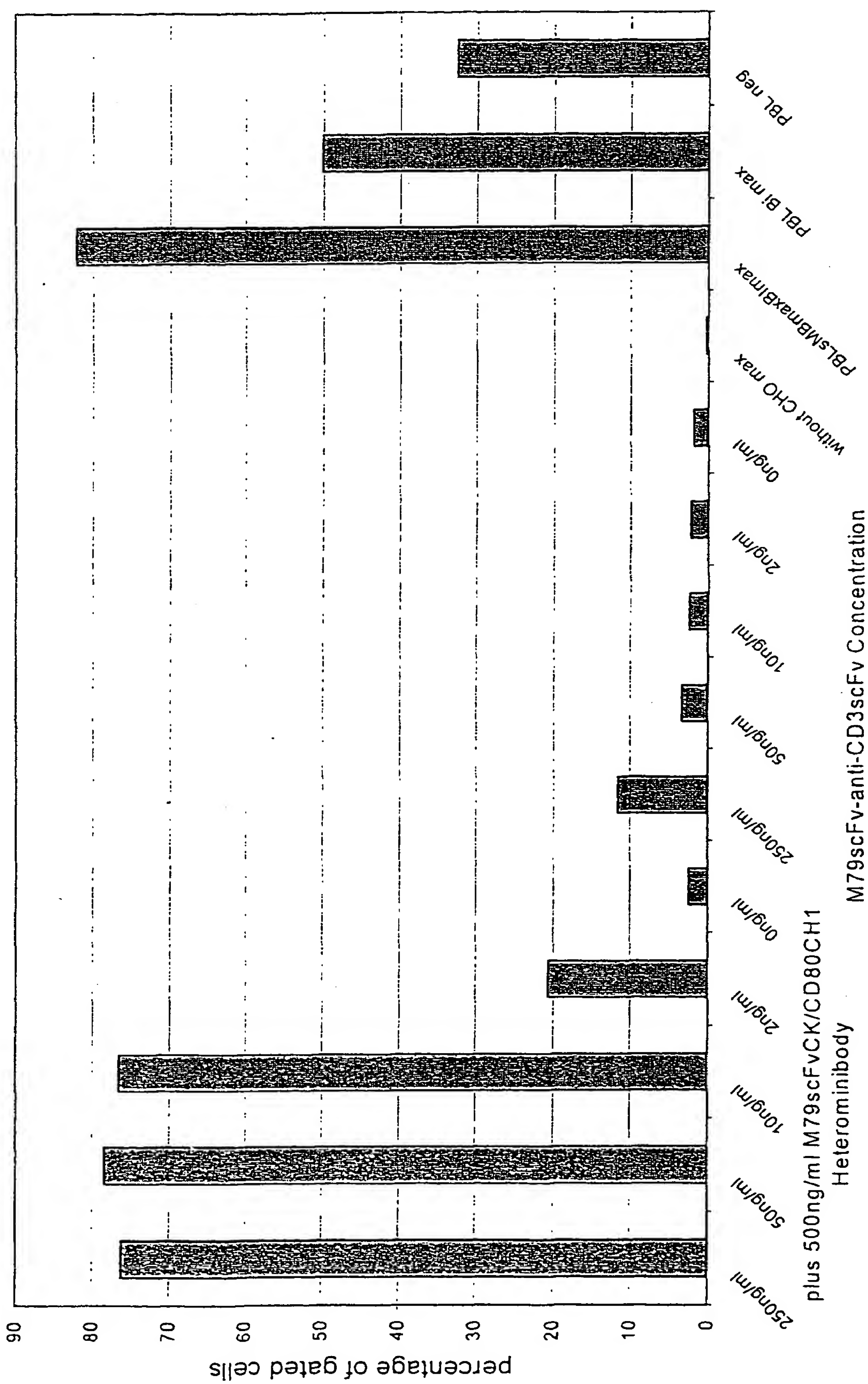
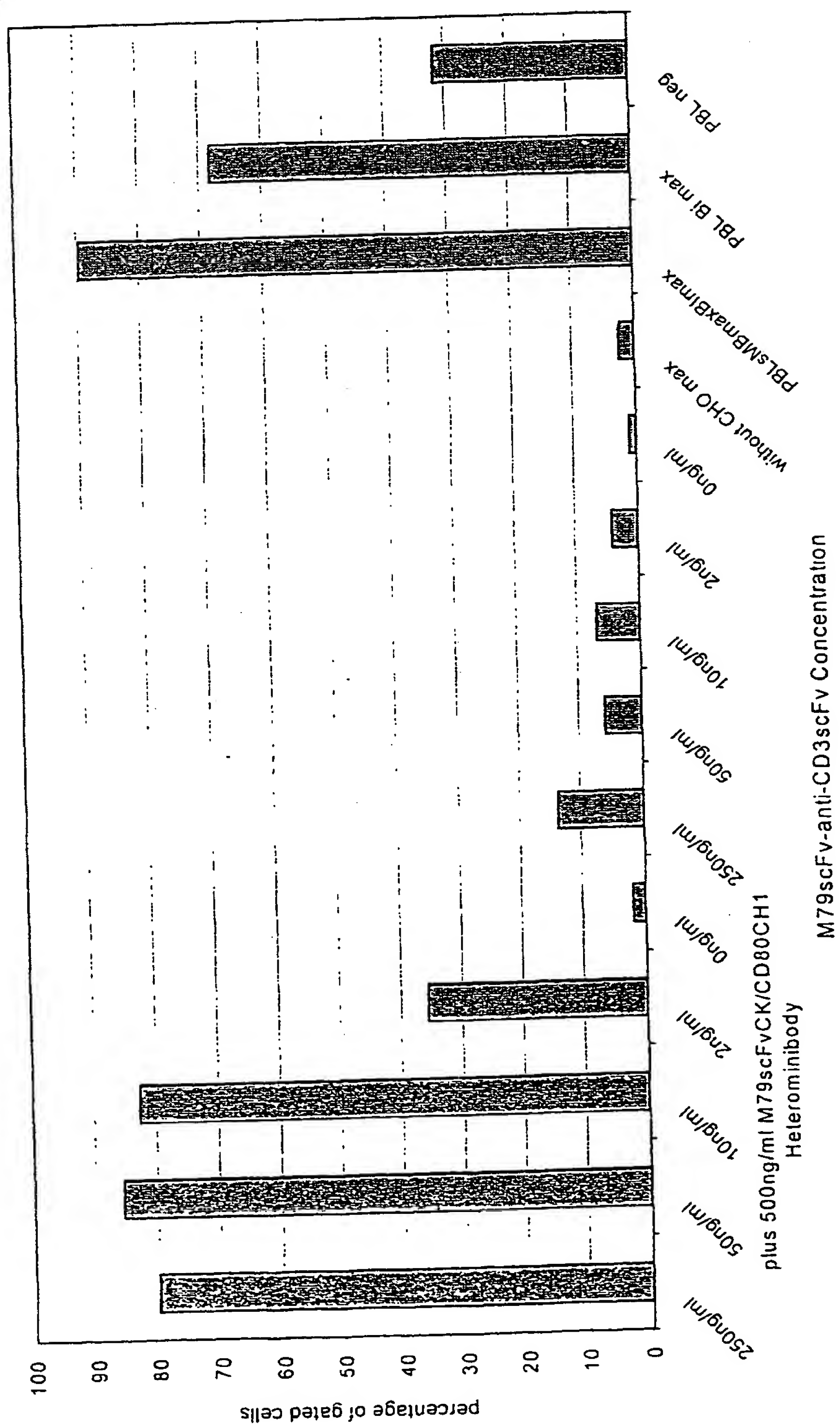

percentage of gated cells
0
10
20
30
40
50
60
70
80
90
PBL neg
PBL Bi max
PBLsMBmaxBimax
without CHO max
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
M79scFv-anti-CD3scFv Concentration
plus 500ng/ml M79scFvCK/CD80CH1 Heterominibody

Figure 20

Figure 21


percentage of gated cells
100
90
80
70
60
50
40
30
20
10
0
PBL neg
PBL Bi max
PBLsMBmaxBimax
without CHO max
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
0ng/ml
2ng/ml
10ng/ml
50ng/ml
250ng/ml
plus 500ng/ml M79scFvCK/CD80CH1
Heterominibody
M79scFv-anti-CD3scFv Concentration

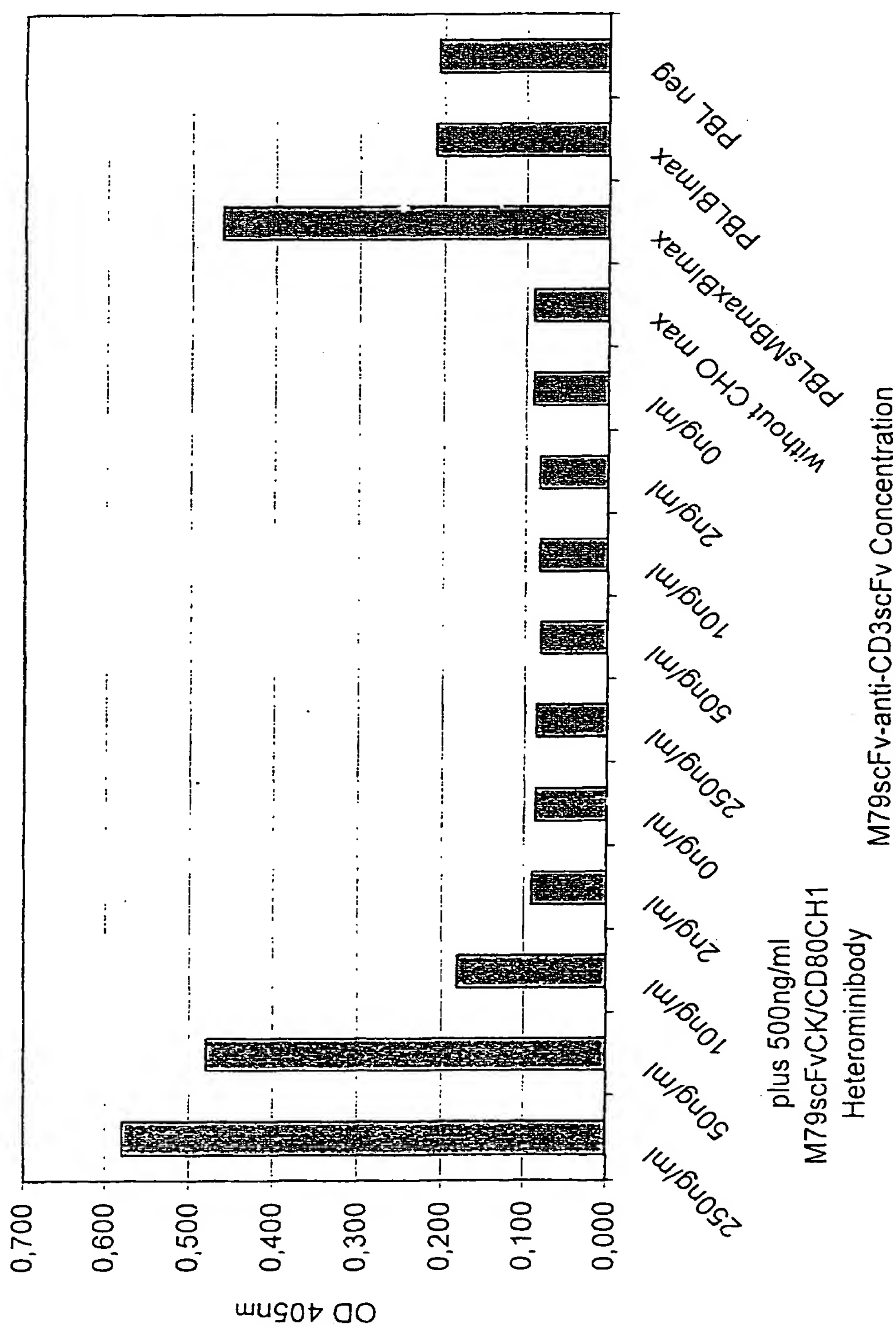

OD 405nm
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
250ng/ml
50ng/ml
10ng/ml
2ng/ml
0ng/ml
without CHO max
PBLsMBmaxBlmax
PBLBlmax
PBL neg
plus 500ng/ml
M79scFvCK/CD80CH1
Heterominibody
M79scFv-anti-CD3scFv Concentration

Figure 22

Figure 23

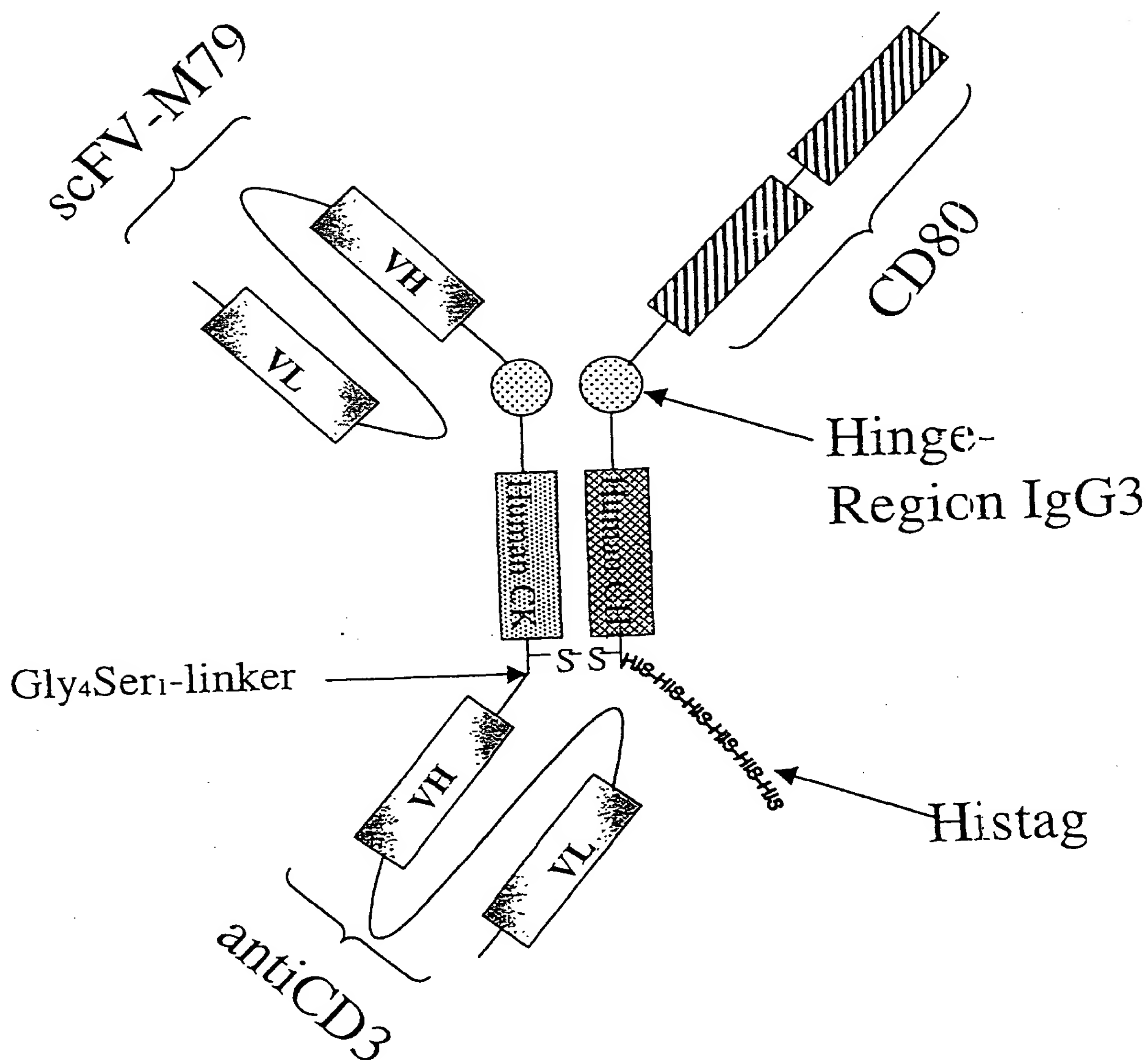

scFV-M79
VH
VL
CD80
Hinge-
Region IgG3
Human CK
S S
Gly4Ser1-linker
VH
VL
Histag
antiCD3

Figure 24

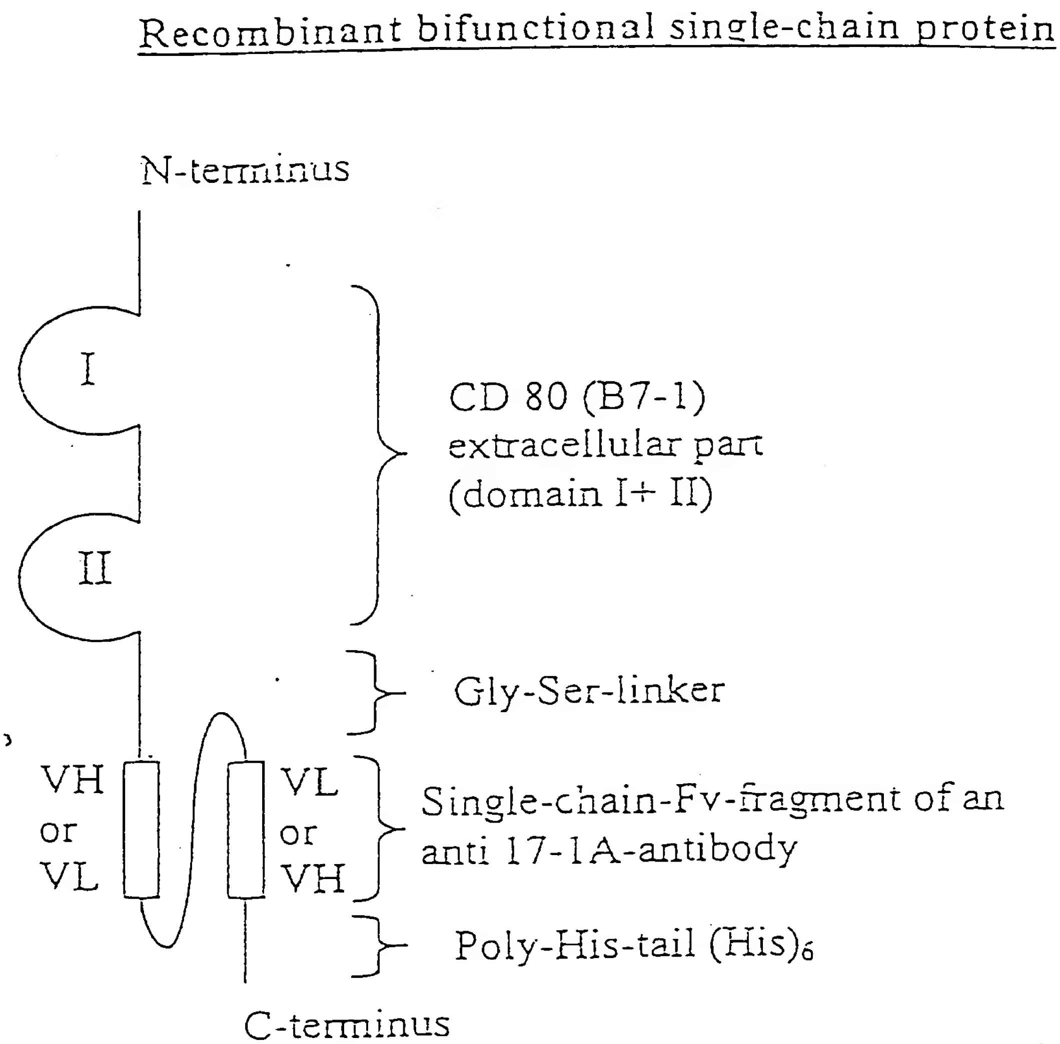

Recombinant bifunctional single-chain protein
N-terminus
I
II
CD 80 (B7-1)
extracellular part
(domain I+ II)
Gly-Ser-linker
VH
or
VL
VL
or
VH
Single-chain-Fv-fragment of an
anti 17-1A-antibody
Poly-His-tail (His)6
C-terminus

Figure 25

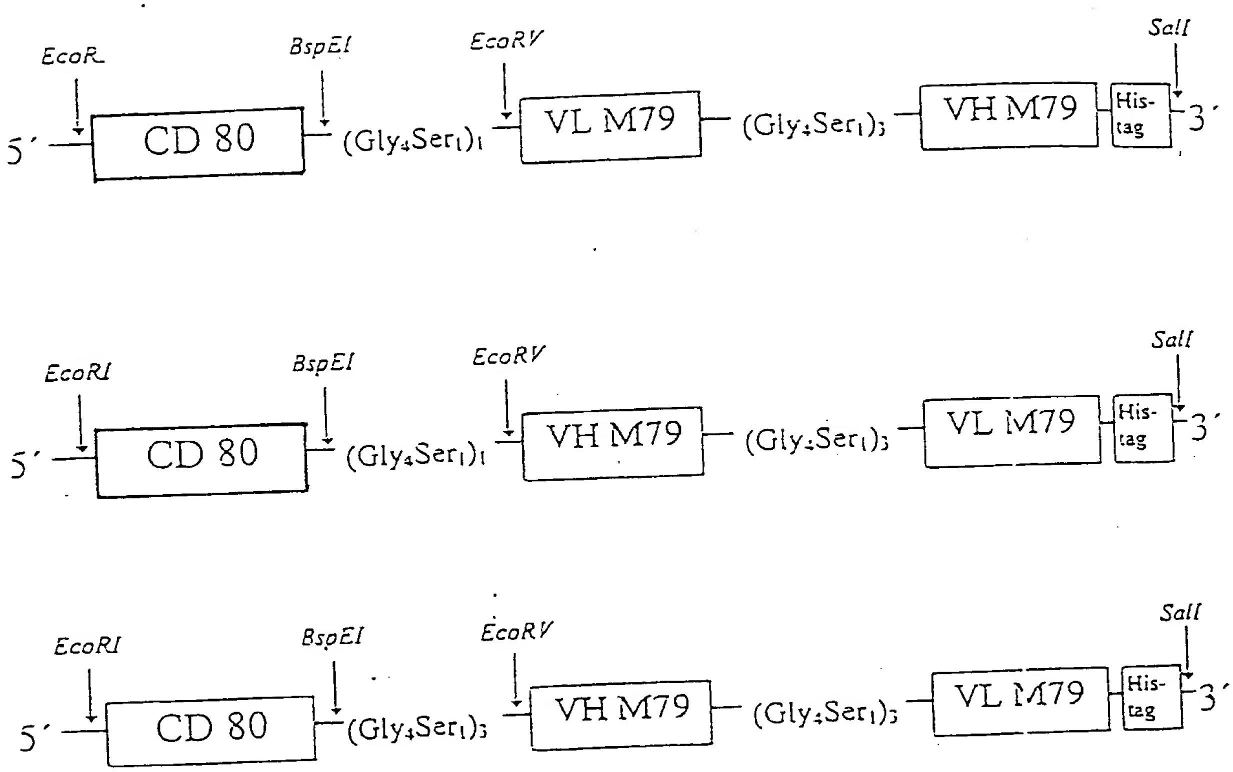

EcoR
BspEI
EcoRV
SalI
5´
CD 80
(Gly4Ser1)1
VL M79
(Gly4Ser1)3
VH M79
His-tag
3´
EcoRI
BspEI
EcoRV
SalI
5´
CD 80
(Gly4Ser1)1
VH M79
(Gly4Ser1)3
VL M79
His-tag
3´
EcoRI
BspEI
EcoRV
SalI
5´
CD 80
(Gly4Ser1)3
VH M79
(Gly4Ser1)3
VL M79
His-tag
3´

Figure 26

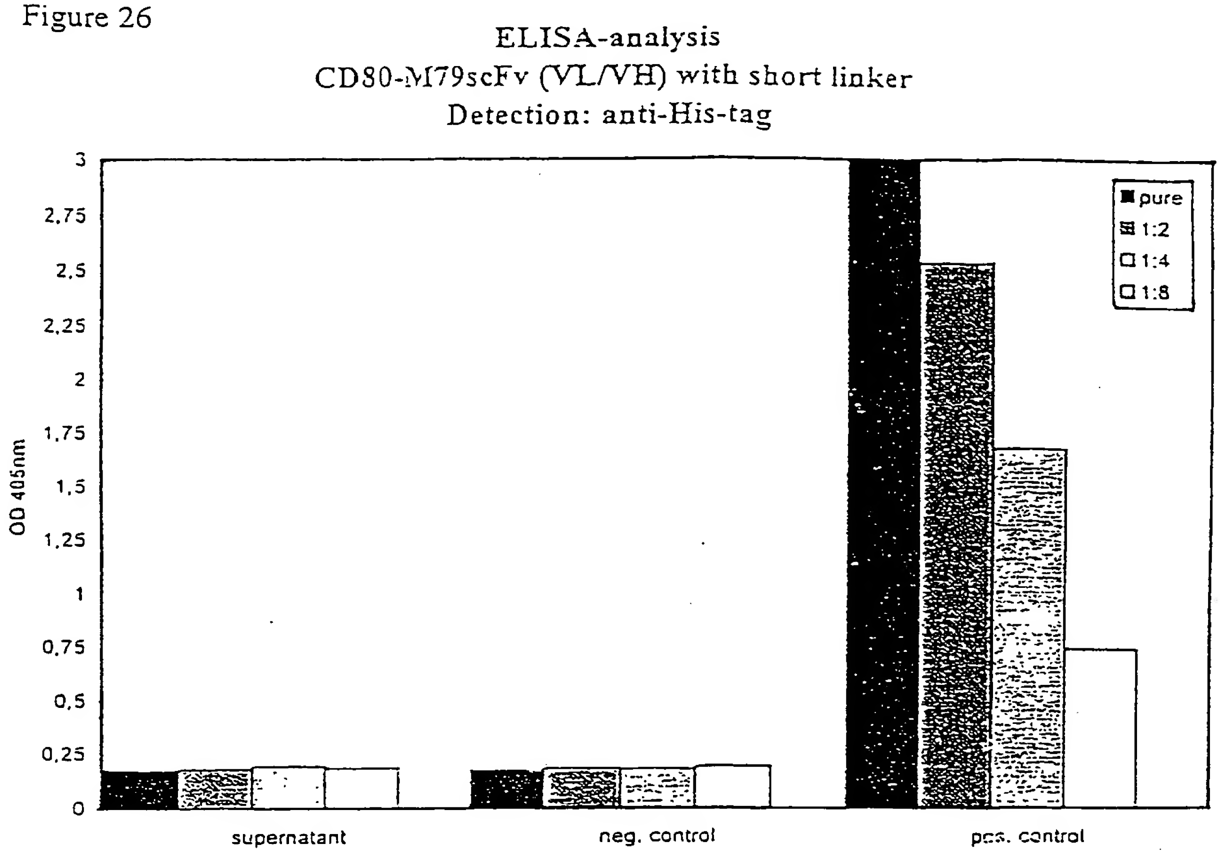


Figure 27

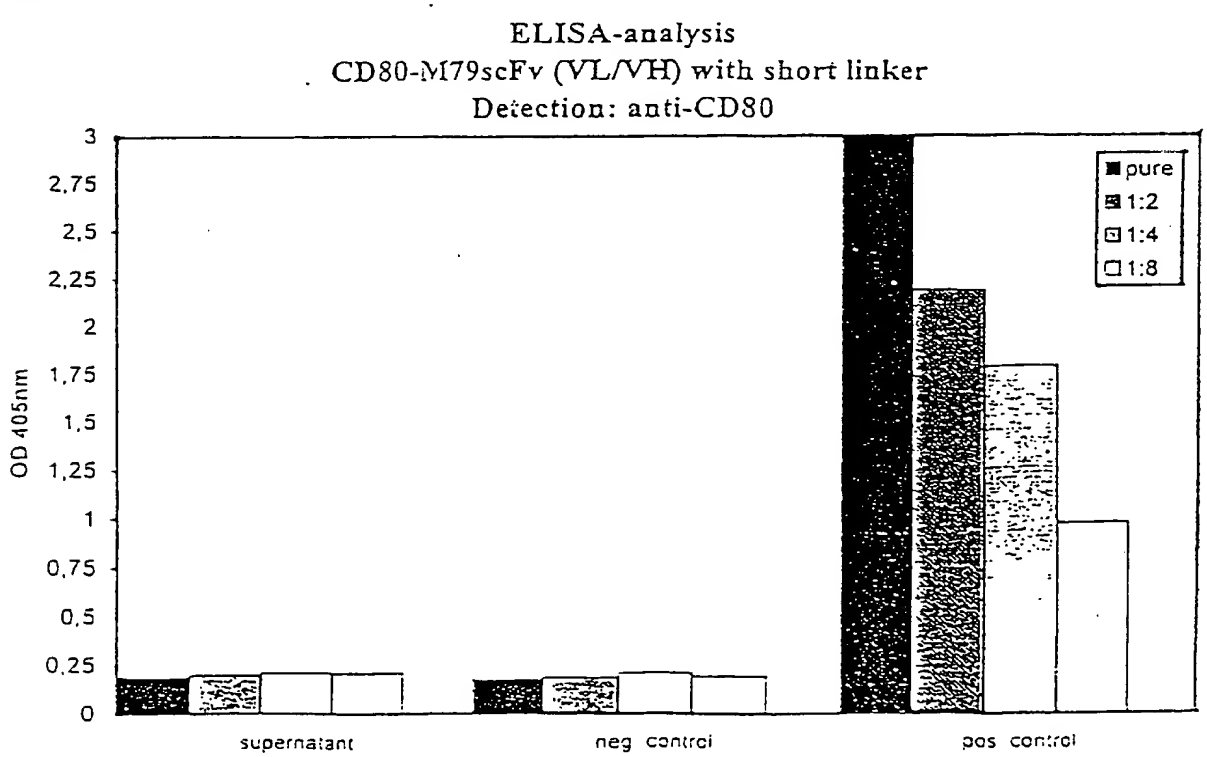


Figure 28

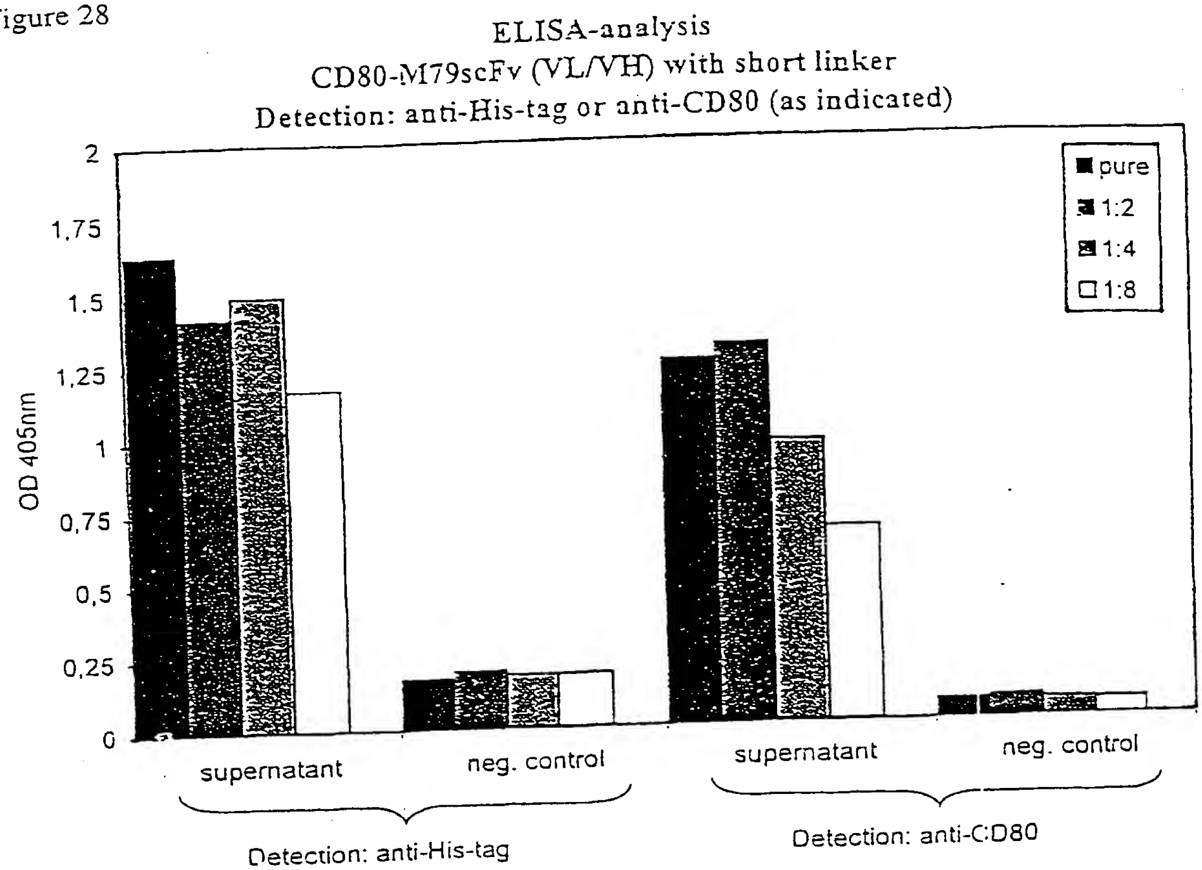


Figure 29

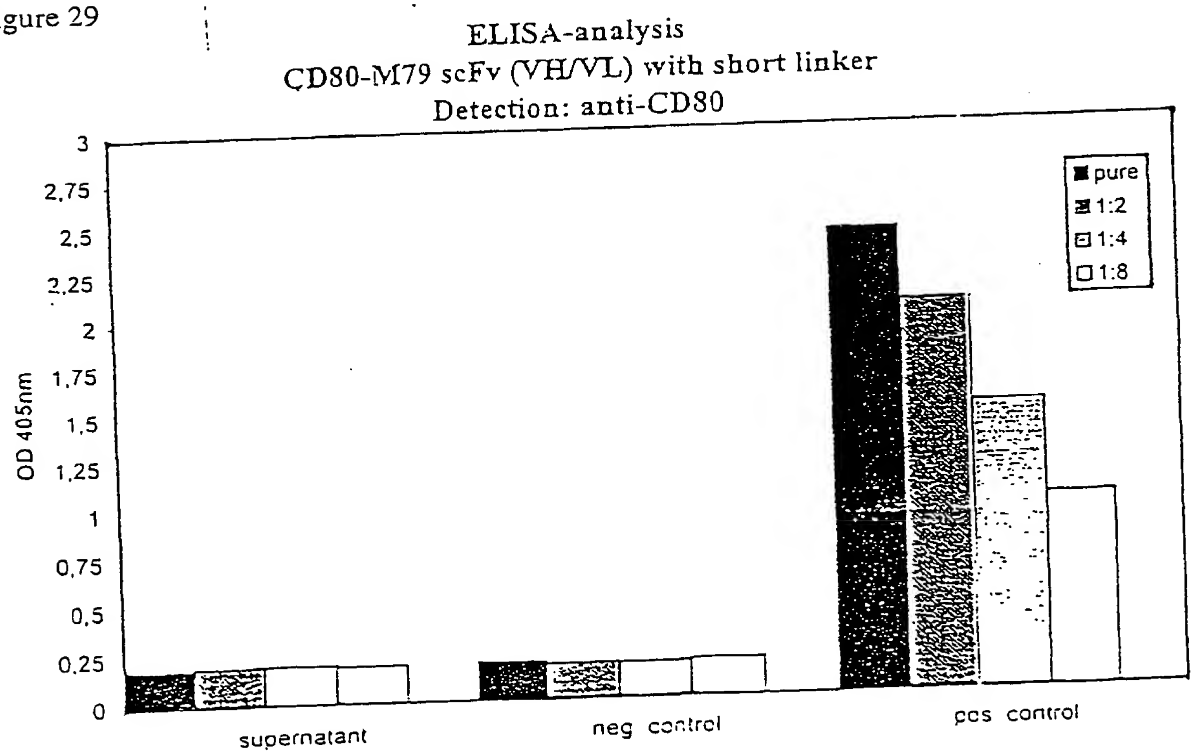


Figure 30

DNA-sequence of the double-stranded oligonucleotide designated ACCGS15BAM

```
     BspEI
        Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
5' pCC GGA GGT GGT GGT TCC GGG GGT GGA GGT TCA GGC GGT GGT G 3'
3'       T CCA CCA CCA AGG CCC CCA CCT CCA AGT CCG CCA CCA CCTAG 5'
                                                          BamHI
```

Figure 31

ELISA-analysis
CD80-M79scFv (VH/VL) with long linker
Detection: anti-His-tag

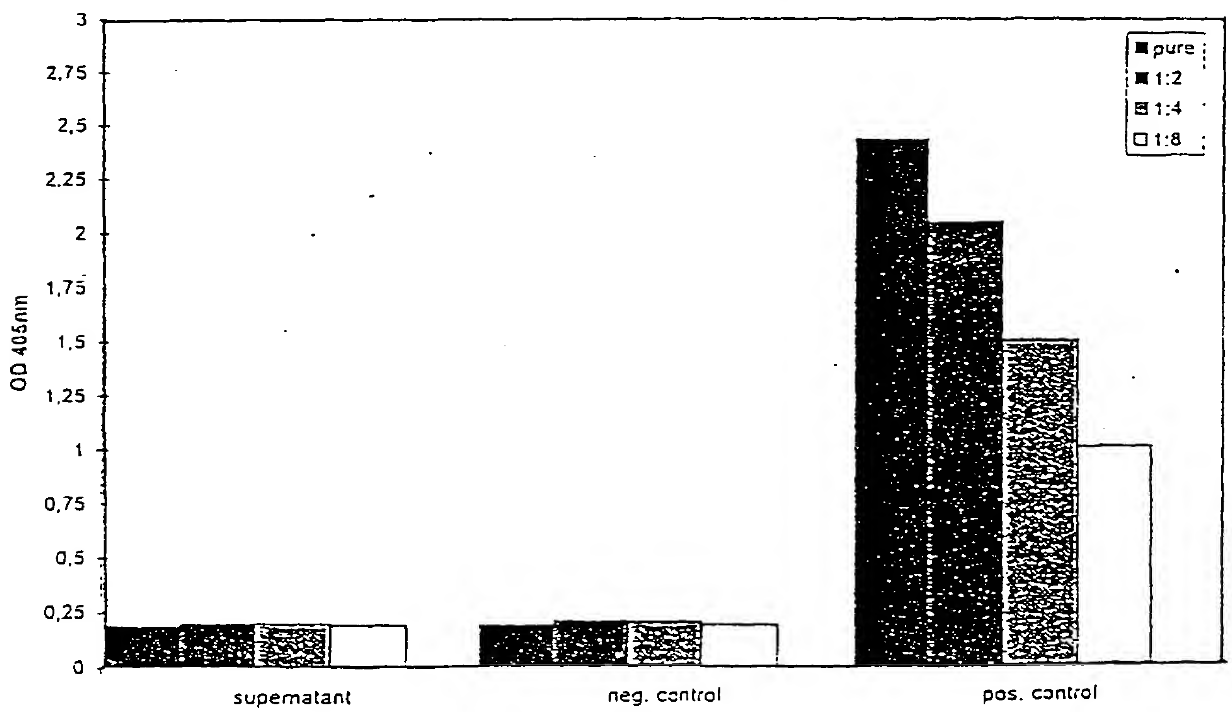
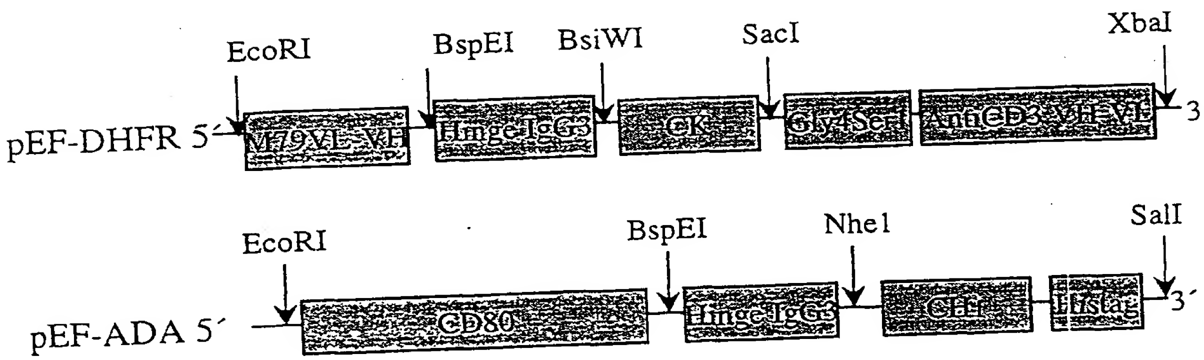


Figure 32


EcoRI
BspEI
BsiWI
SacI
XbaI
pEF-DHFR 5´
M79VL-VH
Hinge IgG3
CK
Gly4Ser1
AntiCD3 VH-VL
3´
EcoRI
BspEI
Nhe1
SalI
pEF-ADA 5´
CD80
Hinge IgG3
CH
Histag
3´